STRATEGIES FOR PREVENTION AND TREATMENT OF HEPATITIS C VIRUS INFECTION

By

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ABSTRACT

Hepatitis C virus (HCV) is the causative agent of Hepatitis C, a serious global health problem which results in liver cirrhosis and hepatocellular carcinoma. Currently there is no effective treatment or vaccine against the virus. Therefore, development of a therapeutic vaccine is of paramount importance. In this project, three alternative approaches were used to control HCV including a DNA vaccine, a recombinant viral vaccine and RNA interference.

The first approach was to test the effect of different promoters on the efficacy of a DNA vaccine against HCV. Plasmids encoding HCV-NS3 and E1 antigens were designed under three different promoters, adenoviral E1A, MLP, and CMV ie. The promoter effect on the antigen expression in 293 cells, as well as on the antibody level in immunized BALB/c mice, was evaluated. The results showed that the antigens were successfully expressed from all vectors. The CMV ie promoter induced the highest antigen expression and the highest antibody level.

Second, the efficiency of a recombinant adenovirus vaccine encoding HCV-NS3 was compared to that of a HCV-NS3 plasmid vaccine. The results showed that the recombinant adenovirus vaccine induced higher antibody levels as compared to the plasmid vaccine.

The relationship between the immune response and miRNA was also evaluated. The levels of mir-181, mir-155, mir-21 and mir-296 were quantified in the sera of immunized animals. mir-181 and mir-21 were found to be upregulated in animals injected with adenoviral vectors.

Third, two recombinant adenoviruses encoding siRNAs targeting both the helicase and protease parts of the NS3 region were tested for their ability to inhibit NS3 expression. The results showed that the siRNA against protease was more effective in silencing the HCV-NS3 gene in a HCV replicon cell line. This result confirmed the efficiency of adenovirus for siRNA delivery.

These results confirmed that CMV ie is optimum promoter for immune response induction. Adenovirus was shown to be an effective delivery vector for antigens or siRNAs. In addition, miRNAs were proved to be involved in the regulation of immune response.

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LIST OF ABBREVIATIONS

Ad	Adenovirus/ Adenoviral
APC	Antigen presenting cell
AP-1	Activator protein-1
Arf1	ADP-ribosylation factor 1
BSA	Bovine serum albumin
CD	Cluster of Differentiation
CMV ie	Cytomegalovirus immediate early
CPE	Cytopathic effect
CpG	Cytosine-phosphate-guanine
CT	Cycle threshold
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
dNTP	Deoxyribose nucleoside triphophate
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
gp E1	Glycoproteins E1
HBV	Hepatitis B virus
HCV	Hepatitis C Virus
HEK 293	Human embryonic kidney 293
HRP	Horse radish peroxidase
Huh-7	Human hepatoma cells
HVR	Hypervariable region
IFN	Interferon
i.m.	Intramuscular
i.p.	Intrapretoneal

IPS-1	IFN-β promoter stimulator-1
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IRES	Internal ribosome entry site
IRF	Interferon regulatory factor
ISDR	IFN sensitivity-determining region
ISRE	IFN-stimulated Response Element
ITR	Inverted terminal repeat
LB	Luria-Bertani media
L-SIGN	Lymph node-specific intercellular adhesion molecule-3-grabbing integrin
MDA-5	Melanoma differentiation-associated gene-5
MHC	Major histocompatibility complex
miRNA	MicroRNA
MLP	Major late promoter
MOI	Multiplicity of infection
MVA	Modified vaccinia ankara virus
NANB	Non-A, non-B Hepatitis
NF-κB	Nuclear factor kappa B
NK	Natural killer
NCR	Non coding region
NOD	Nucleotide oligomerization domain
nt	Nucleotide
OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffered saline
PFU	Plaque forming unit
RdRP	RNA dependent RNA polymerase
RIG-1	Retinoic acid-inducible gene-I
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNase	Ribonuclease
RLR	RIG-1 like receptors
RT	Reverse transcription
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	Short hairpin RNA

siRNA	Small interfering RNA
SOCS3	Suppressor of cytokine signaling 3
STAT	Signal transducers and activators of transcription
SV40	Simian virus 40
SVR	Sustained virological response
TA	Tibialis anterior
Th	T Helper cells
TLR	Toll-Like receptor
Treg	Regulatory T cells
TRIF	TIR-domain-containing adaptor-inducing IFN-β
UTR	Untranslated region
VLP	Virus-like particles

PROJECT OVERVIEW

1- Background

Hepatitis C virus (HCV) constitutes a major global concern that affects more than 170 million individuals worldwide. Chronic HCV infection usually progresses to liver cirrhosis and hepatocellular cancer, resulting in a high annual mortality rate (Saito *et al.*, 1990). Currently, there is no effective treatment or vaccine against the virus. This is due in large part to the high mutation rate of HCV, enabling the virus to escape the body's natural immune responses, as well as immunity acquired by vaccinations. The genomic mutations also contribute to the virus's ability to resist therapy. Therefore, development of an effective therapeutic vaccine is of paramount importance both for the prevention and treatment of HCV.

In order for a vaccine to be effective in preventing chronic HCV infection, it must trigger an early, strong immune response. Efficient antigen expression and delivery are important for the induction of a potent immune response. Although traditional vaccines based on inactivated or attenuated infectious agents are often effective, they run the risk of retrieving the wild type virus. This risk increases when the mutation rate is high, as is the case for HCV. This has led to the development of novel vaccine approaches. During the last two decades, DNA-based vaccination has gained attention due to its ability to produce both humoral and cellular immune responses, as well as its ease of use, safety, and low cost of preparation. However, this approach still requires optimization to be of clinical use. Since antigen expression levels and delivery are two key factors that induce a potent immune response, recombinant viral vaccines, that exhibit enhanced cellular

uptake efficiency, provide another attractive alternative. Finally, new therapeutic approaches including siRNA are being developed that depend on the inhibition of viral replication by silencing their vital enzymes. In this thesis, these three current approaches are explored, evaluated, and compared in order to determine which approach might best be employed to develop an effective vaccine or therapy against HCV.

2- Overall objective

The primary goal of this project is to study strategies for prevention and treatment of HCV. To achieve this goal, three distinct but complementary approaches were used: (1) a plasmid DNA vaccine with different promoters, (2) a recombinant adenoviral vector vaccine, and (3) a small interfering RNA (siRNA) based strategy.

3- Experimental design

The first two approaches developed in this study are HCV vaccines involving the following steps: (1) identify the antigens of interest, (2) engineer a construct consisting of the gene of interest under the control of an appropriate promoter, (3) insert the construct into the vehicle of choice (e.g., plasmid or adenoviral vector) for delivery to the host cell, (4) test the construct for efficacy in cell culture, (5) test the construct for immune response *in vivo* using a small animal model (e.g., mouse), and (6) evaluate and compare the results to optimize the efficacy of the potential HCV vaccine. The third approach used in this study is an evaluation of an HCV vaccine/therapy that combines the adenoviral vector delivery system with siRNA as a means to target and inactivate the critical viral enzymes, helicase and protease.

4- Chapter overviews

In Chapter 1, a literature review is conducted describing the HCV life cycle, pathogenesis, biology, and treatments. The human immune response to infection is also discussed. Current approaches in vaccine development are highlighted. Methods aimed at eliciting and enhancing the immunological response to HCV infection are introduced, and additional strategies to target and clear the virus are discussed.

Chapter 2 describes the development of plasmid DNA vaccines using two specific HCV antigens: NS3 and E1. In order to optimize the antigen expression levels for NS3 and E1, the effect of promoter strength on vaccine efficacy was tested and evaluated using three promoters: (1) cytomegalovirus immediate early promoter (CMV ie), (2) adenoviral early E1A promoter (E1Ap), and (3) adenoviral major late promoter (MLP). Promoter strength was measured by transcription levels and antibody production in cell culture and mouse models respectively. In addition, changes in mouse miRNA serum levels after immunization were evaluated for three miRNAs known to be involved in the immune response: (1) miRNA-21, (2) miRNA-181, and (3) miRNA-296. Promising directions for further vaccine development are also discussed.

Chapter 3 describes the construction of a recombinant adenoviral vector vaccine against HCV NS3 (rAdNS3) under the control of the CMV ie promoter. Construct efficacy, measured as antigen transcription efficiency, is evaluated in cell culture (HeLa cells) and antibody production was evaluated in mice. Mouse serum miRNA -21, -181, and -296 levels are also measured and the results are compared to those observed for the plasmid DNA vaccine developed in chapter 2.

In Chapter 4, an alternative strategy was utilized to evaluate the efficacy of RNA interference (RNAi) against HCV-NS3 as a therapeutic tool. Two short hairpin RNAs (shRNA) expressed by adenovirus were constructed to target the HCV-NS3 region, which encodes two enzymes that are critical to HCV replication: protease and helicase. The constructs were tested in the animal model system, to test infecting hepatoma cells expressing the HCV 1b genome (Con1-FL/Neo) with each AdshRNA. NS3 RNA was quantified using qRT-PCR and the results are discussed.

Finally a general conclusion of the overall results and trends that emerge from this thesis is provided. The potential applications of the thesis, the next steps to be taken in vaccine development, and directions for future research are outlined.

5- Key findings

Four key findings emerge from this thesis. First, the CMV ie promoter had the highest level of expression for E1 and NS3 genes, making CMV ie the promoter of choice. Second, all vaccines elicited a specific antibody response and the recombinant adenoviral vector vaccine expressing NS3 induced a higher immune response than the DNA vaccine. Third, certain miRNAs were upregulated in animals injected with adenoviral vectors compared to plasmid DNA vaccines, providing additional support for the efficacy of the viral vector vaccine. This finding also points to these miRNAs as potential indicators of immune response activation. Fourth, siRNA against HCV protease was effective in silencing the HCV-NS3 gene. This suggests that these approaches are promising for both prevention and treatment of HCV, and warrant further investigation.

Chapter 1: LITERATURE REVIEW

1.1- Introduction

HCV is a major health concern that leads to liver failure affecting more than 170 million people, representing around 3% of the world's population (Wasley and Alter, 2000). The virus is the causative agent of hepatitis C, a liver-associated disease. HCV is transmitted by blood and to a lesser extent through sexual and maternal routes. Although other hepatitis viruses exist, vaccines are available for both hepatitis A and hepatitis B but not for HCV, making the latter the leading cause for liver disease-associated death and the primary reason for liver transplantation. HCV infection has spread in developing countries due to unsafe medical practices. Although infection through blood transfusion has decreased significantly in developed countries since 1992 when blood screening for HCV became available, the prevalence of the infection remains high as those previously infected only start to show serious symptoms years after infection. The prevalence of infection is also elevated in high risk groups including intravenous drug users and HIV patients. Coinfection with HIV and HCV accelerates the progression to liver cirrhosis and failure (Weber *et al*., 2006). Since no effective vaccine is available, the virus remains a serious global health threat. Even with the available therapy based on a combination of IFN and ribavirin, complete resolution of the virus is not achieved in most infected patients. Thus, novel therapeutic approaches are being investigated, such as inhibitors and RNAi against viral enzymes. Moreover, there is currently no vaccine against HCV due in part to the heterogeneity of the virus. Extensive research focuses on developing vaccines against the virus and some vaccines have been evaluated in clinical trials. In the

sections that follow, a literature review highlighting current knowledge in the field will be provided. First, the biology and immunology of HCV and progress in virus control will be discussed. Second, different vaccine and therapeutic approaches against HCV will be introduced.

1.2- Hepatitis C virus discovery

HCV was first discovered in the seventies in post transfusion hepatitis cases. The new agent at the time was clinically and histologically similar to the two known hepatitis viruses, A and B, but lacked the serological markers of the latter viruses. Therefore, the yet unknown agent was referred to as non-A, non-B (NANB) Hepatitis (Feinstone *et al.*, 1975). In the late 80s the genome of the infectious agent was isolated and cloned, and the virus was identified as HCV (Choo *et al.*, 1989). Based on the structural and virological characteristics, HCV was classified in the Flaviviridae family, which is divided into three genera: flavivirus, pestivirus, and hepacivirus (Rosenberg, 2001). The Hepacivirus genus includes HCV and another two members, tamarin virus and GB virus B (Lindenbach and Rice, 2001).

1.3- Hepatitis C Virus pathogenesis

HCV infects hepatocytes and is transmitted mainly by blood contact. To date, there are more than 170 million people chronically infected with HCV throughout the world. Only 15% of infected people develop a strong immune response capable of eliminating the infection during the acute phase, which lasts for about 6 months while more than 85% enter the chronic phase, during which the virus persists and replicates in the liver. The infection remains asymptomatic for around 20-30 years before it progresses

to liver cirrhosis, liver failure, or hepatocellular carcinoma (Alter, 1995; Levrero, 2006). The asymptomatic nature of the infection contributes to the increasing spread of the virus, as infected people may not be aware of their infection until a later stage.

1.4- Hepatitis C virus structure and genome

HCV is a small, spherical, enveloped virus of 55-65 nm in diameter (Kaito *et al.*, 1994). Under the envelope, a protein capsid surrounds the positive single-stranded RNA genome. Glycoproteins are embedded in the outer envelope. HCV structure is illustrated in Figure 1.1. The HCV genome consists of a long open reading frame (ORF) of ~9600 nucleotides (nt). The coding region is flanked by the 5' and 3' untranslated regions (UTR). The 5'UTR is a highly conserved region of approximately 340 nt. It has a strong secondary structure and contains the internal ribosomal entry site (IRES) that mediates ribosome binding, and translation initiation (Pilipenko *et al.*, 1989). The 3'UTR is a less conserved and less structured region of about 200 nt. It contains a poly-uridyl tract, with various lengths in different strains, which was found to be essential for viral RNA replication (Friebe and Bartenschlager, 2002; Yi and Lemon, 2003a; 2003b). The genome is translated into a single polyprotein precursor of ~3000 amino acids, which is co- and post-translationally processed by cellular peptidases and viral proteases to produce three structural and six non-structural viral proteins in addition to the p7 protein (reviewed in Penin *et al.*, 2004). The structural proteins are cleaved with cellular peptidases, releasing the core protein which forms the nucleocapsid surrounding the viral genome as well as the envelope glycoproteins 1 and 2 (E1 and E2) that are involved in the attachment and entry into the host cell. The nonstructural proteins, designated NS2, NS3, NS4A, NS4B,

NS5A, and NS5B are released by the viral proteases (Dubuisson *et al.*, 2002). These proteins are involved in viral replication and host immune evasion (Lai, 2000).

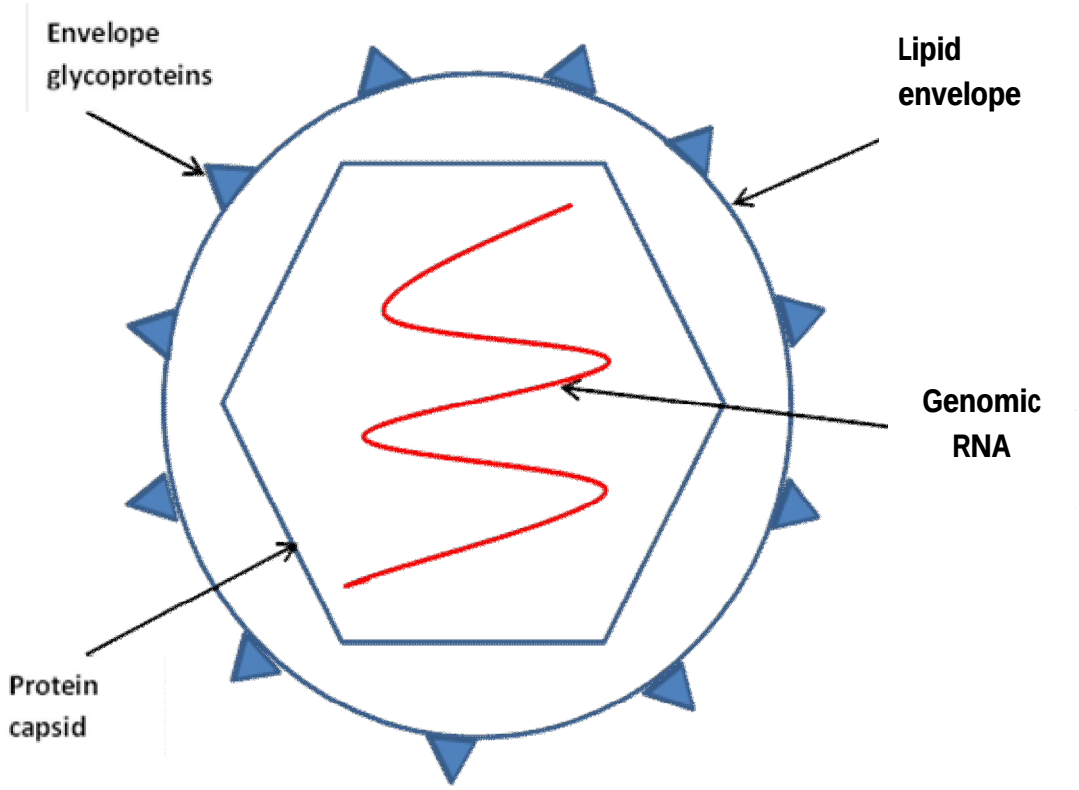


Figure 1.1: Schematic diagram of the structure of HCV.

1.5- Hepatitis C virus proteins

The core protein is a highly conserved multifunctional protein. Aside from its structural function in forming the viral capsid, it is implicated in viral replication, maturation, and pathogenesis (Suzuki and Suzuki, 2006). E1 and E2 glycoproteins are involved in receptor binding and entry into the host cell. E1 and E2 form a non-covalent heterodimer which assists in viral attachment. E2 is the most variable region in the viral

polyprotein as it contains two hypervariable regions (HVR) (Op De Beeck *et al.,* 2001; Cocquerel *et al.,* 2002). P7 is a small hydrophobic protein forming an ion channel embedded in the endoplasmic reticulum (ER) membrane (Griffin *et al.,* 2003; Pavlovic *et al.,* 2003). P7 is essential for RNA replication, viral assembly, and release (Jones *et al.,* 2007; Steinmann *et al.,* 2007). NS2 together with the N' terminal domain of NS3 forms a zinc-stimulated autoprotease that cleaves the NS2/NS3 junction (Grakoui *et al.,* 1993; Hijikata *et al.,* 1993). NS3 is a large multifunctional protein with enzymatic activities that are essential for viral RNA replication and polyprotein processing. The N' terminal portion of NS3 (180 amino acids) is a serine protease which works with cofactor NS4A to cleave the downstream junctions and release NS proteins. The rest of the protein comprises the helicase-NTPase domain, which unwinds RNA during replication (Kolykhalov *et al.,* 2000; Lam and Frick, 2006). The function of NS4B is not clear, but it is thought to have a role in the formation of HCV membranous replication complexes (Egger *et al.,* 2002; Gao *et al.,* 2004). NS5A is a phosphoprotein which has a role in the regulation of RNA replication (Evans *et al.,* 2004; Gao *et al.,* 2004), as well as inhibition of the host immune response (Kriegs *et al.,* 2009). NS5B is an RNA dependent RNA polymerase (RdRP). It is a highly conserved protein which initiates the synthesis of new RNA strands (Schmidt-Mende *et al.,* 2001; Moradpour *et al.,* 2004). NS5B lacks proof reading ability, thus it is prone to a high mutation rate. This contributes to the high genetic diversity of HCV, enabling the virus to escape the host immune response, often leading to treatment failure. Figure 1.2 shows the HCV genome and encoded proteins.

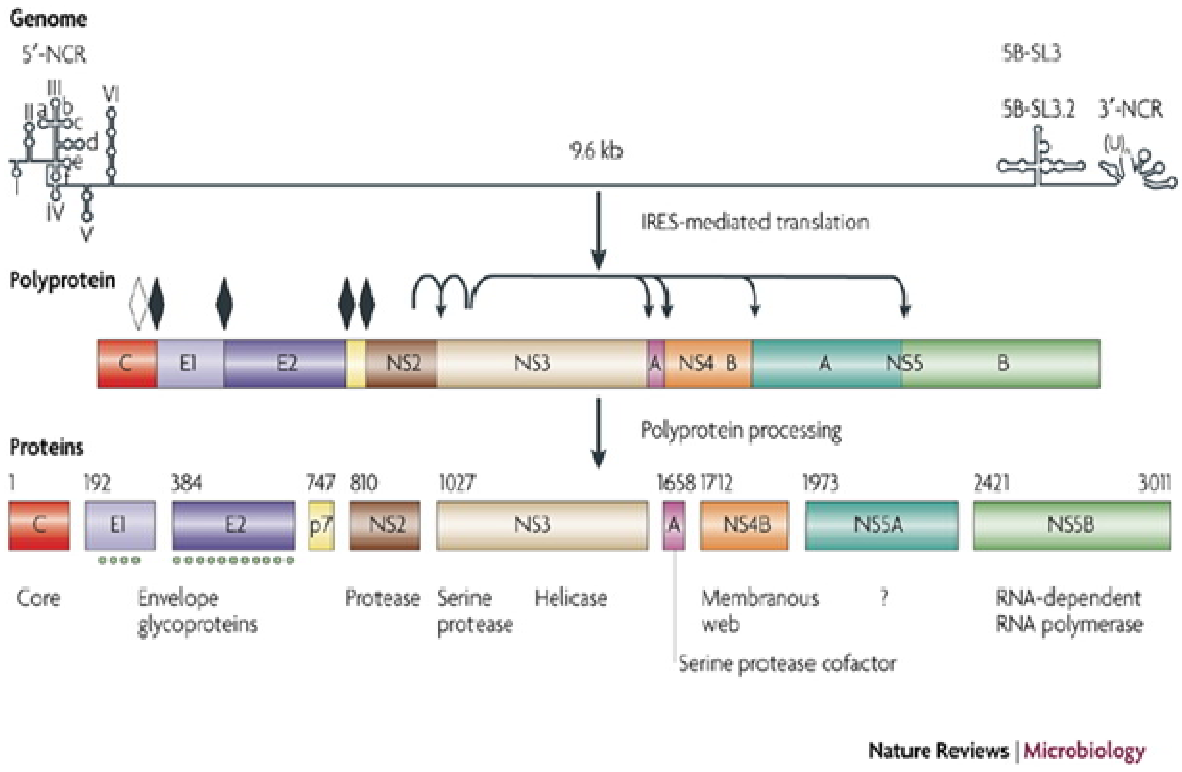


Figure 1.2: HCV genome structure and protein processing. (Moradpour *et al.*, 2007).

1.6- Hepatitis C virus life cycle

HCV replicates in the hepatocytes (Shimizu *et al.*, 1990; Nouri-Aria *et al.*, 1995). The HCV infection cycle consists of five steps: attachment and entry, protein translation and processing, genome replication, viral assembly and exit. The first step is attachment and entry into the host cell. Different cell surface molecules are thought to be involved in HCV attachment, and work together to facilitate viral entry into host cells. These receptors include: Cluster of Differentiation 81 (CD81) (Pileri *et al.*, 1998), the human scavenger receptor class B type I (SR-BI) (Scarselli *et al.*, 2002), the liver and lymph node-specific intercellular adhesion molecule-3-grabbing integrin (L-SIGN), the dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN)

(Gardner *et al.,* 2003; Lozach *et al.,* 2003; Pohlmann *et al.,* 2003) and a tight junction component claudin-1 (Evans *et al.,* 2007). The viral proteins E1 and E2 bind to cellular complex receptors and the virus enters the cell by clathrin-mediated endocytosis (Bartosch *et al.,* 2003; Nielsen *et al.,* 2004). Following entry, the fusion of endosomal and viral membranes leads to the release of viral RNA into the cytoplasm (Moradpour *et al.,* 2007).

The positive sense viral RNA is immediately translated using the cellular machinery in a cap-independent manner, mediated by the IRES (Luo *et al.,* 2003). Translation is initiated in the cytoplasm, where the polyprotein is processed, producing the individual proteins. The mature proteins localize to the ER where the NS proteins and viral RNA form the replication complex (Penin *et al.,* 2004; Egger *et al.,* 2002). Replication is performed by the viral RdRP, through a negative-strand RNA intermediate (Bartenschlager *et al.,* 2004). The new RNA strands, with the structural protein, combine to form new viral particles which acquire an envelope by budding through the ER membranes. The viral particles are then released from the cell by exocytosis (Dash *et al.,* 1997; Mizuno *et al.,* 1995). HCV is not a lytic virus, and does not lead to cell death. The life cycle of HCV is shown in Figure 1.3.

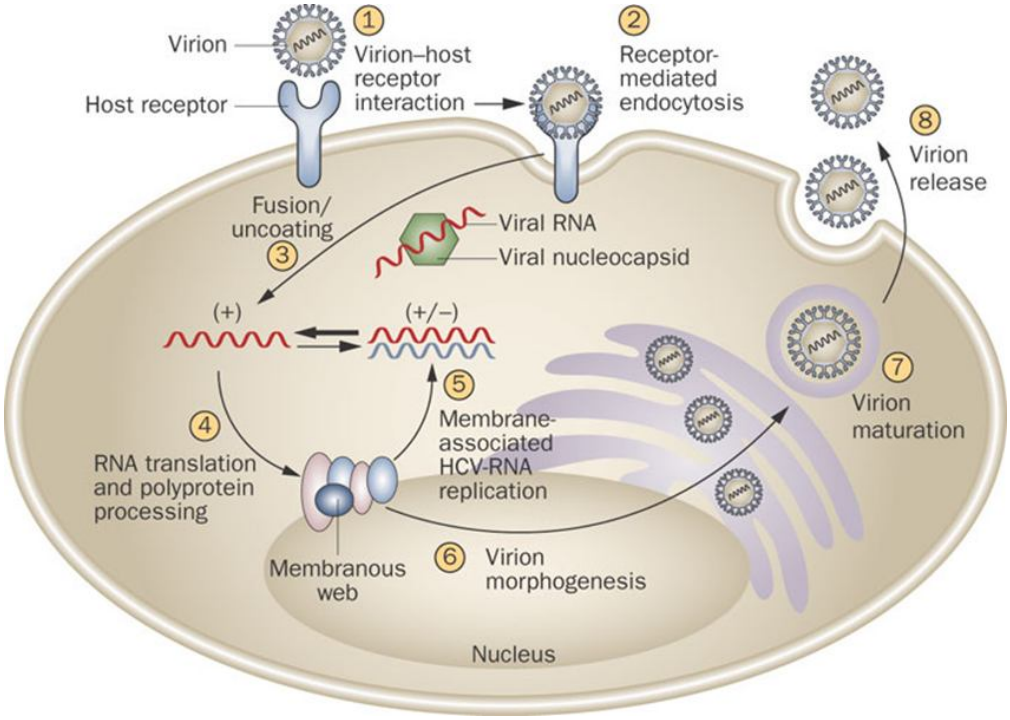


Figure 1.3: The life cycle of HCV (Pereira and Jacobson, 2009).

1.7- Hepatitis C virus genotypes

HCV has an extremely heterogeneous genome, due to rapid replication and the poor fidelity of its RNA polymerase. It goes through 1.5–2.0 x 10^{-3} nt substitutions per site per year (Bukh *et al.*, 1995). Based on its genetic variability, HCV is classified into six major genotypes designated 1 to 6, which further divide into multiple subtypes (Robertson *et al.*, 1998; Simmonds, 1995). Furthermore, infected hosts may harbor multiple virus genotypes, which are referred to as a quasispecies (Domingo *et al.*, 1985; Weiner *et al.*, 1992; Wyatt *et al.*, 1998). The various genotypes differ in their geographical distribution (Bukh *et al.*, 1995). For instance, HCV genotype 1 is predominant in Europe and North America, while genotypes 2, 4 and 5 are found in Africa (Candotti *et al.*, 2003; Verbeeck *et al.*, 2006). Genotype 6 is the main strain found

in Asia. Egypt has the highest prevalence of HCV infection predominated by genotype 4. Almost 20% of the Egyptian population is infected with HCV (Ray, 2000; Frank *et al.,* 2000; Tanaka *et al.,* 2004). This represents an increasing public health burden in Egypt (Deuffic-Burban *et al.,* 2006). Moreover, the outcome of the alpha interferon (IFN-α) therapy greatly depends on the genotype, with genotypes 1 and 4 being the least responsive (Manns *et al.,* 2001; Fried *et al.,* 2002).

1.8- The Interaction between HCV and host immunity

1.8.1- The host immune response

The human immune system is effective at fighting infectious pathogens. The initial line of defense is the nonspecific innate immunity, which helps control the infection and activates the specific adaptive immunity, including the cellular and humoral immune response. HCV, like any virus, stimulates a series of antiviral pathways however, the persistence of the virus demonstrates its ability to evade the immune responses.

1.8.1.1- Innate immunity

The innate immune response is induced early after infection with HCV. The liver has different kinds of innate immune cells including Kuppfer cells (also known as resting macrophages), circulating natural killer (NK) cells and dendritic cells (DC). These cells, as well as hepatocytes, contain receptors that sense the viral infection through viral recognition patterns, such as the ssRNA and dsRNA produced during the viral replication cycle. These receptors belong to two families, the toll-like receptors (TLRs), which are trans-membrane receptors located in the endosome, and RIG-1 like receptors (RLRs) which are located in the cytoplasm. Activation of these receptors induces IFNs,

key proteins involved in the early control of viral infection (Kanto and Hayashi, 2007). IFNs use multiple redundant pathways to prevent viral spread to neighboring cells (Figure 1.4). This adaptation overcomes the viral defense mechanisms. IFNs activate transcription of several antiviral genes and play a role in the maturation of NK cells, other key players involved in the innate immune response (reviewed in Racanelli and Rehermann, 2006).

1.8.1.1.1- Interferon induction pathways

Viral dsRNA is detected by TLR-3, located in hepatocytes, DCs and macrophages, and initiates signal transduction through the adaptor protein TIR-domain-containing adaptor-inducing IFN-β (TRIF). Stimulation of TLR3 in endosomes and on the cell surface (Eisenacher *et al.,* 2007) recruits IκB kinase (IKK), TANK-binding kinase 1 (TBK1) and IKKi. These kinases, together with adaptor molecules TANK and NAP1, induce the phosphorylation and nuclear translocation of interferon regulatory factor- (IRF) 3 and 7. In the nucleus, phosphorylated IRF-3 and IRF-7 collaborate with transcription factor activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB), to regulate the expression of IFN-β (Asselah and Marcellin, 2011).

An alternative pathway is mediated by cytoplasmic RLR including retinoic acid-inducible gene-I (RIG-1) and the homologous protein melanoma differentiation-associated gene-5 (MDA-5). RIG-1 is triggered by a polyuridine sequence in the 3' non coding region (3'-NCR) (Hornung *et al.,* 2006; Kato *et al.,* 2008) and MDA-5 is triggered by long dsRNA. These sensor proteins bind dsRNA using their C terminal helicase. Through their N terminal CARD domains, a downstream signaling cascade is activated via the mitochondrial adaptor molecule IPS-1 (IFN-β promoter stimulator-1, also known

as CARDIF, MAVS and VISA) (Kawai *et al.,* 2005; Seth *et al.,* 2005; Xu *et al.,* 2005). IPS-1 activates both IRF-3 and IRF-7 resulting in their dimerization and translocation to the nucleus where they induce the transcription of IFN-α/β.

IFN -α/β binds to a common receptor (IFNAR-1/2) expressed at the cell surface of target cells, leading to activation of the JAK/STAT pathway. The activation of the Signal Transducers and Activators of Transcription (STAT) protein is mediated by phosphorylation by the Janus-family tyrosine kinases Tyk2 and Jak1. STAT1 and STAT2 combine with IRF-9 forming the Interferon-Stimulated Transcription gene Factor-3 (ISGF3) complex. The ISGF3 complex binds to the cis-acting IFN-stimulated Response Element (ISRE) in the nucleus initiating transcription of several IFN-inducible genes. These genes include protein kinase R (PKR), and oligoadenylate synthetase (OAS)/RNaseL (Gale and Foy, 2005). RNaseL degrades the viral RNA inhibiting translation (Asselah *et al.,* 2009; 2010). PKR is an integral component in the human innate immune response mechanism. PKR recognizes and is activated by long stretches of dsRNA (Samuel, 2001; Cole, 2007). Subsequently PKR phosphorylates eukaryotic initiation factor 2α (eIF2α), resulting in the shutdown of cellular translation, and inhibition of viral proliferation (Garcia *et al.,* 2006). The IFN pathways are summarized in Figure 1.4.

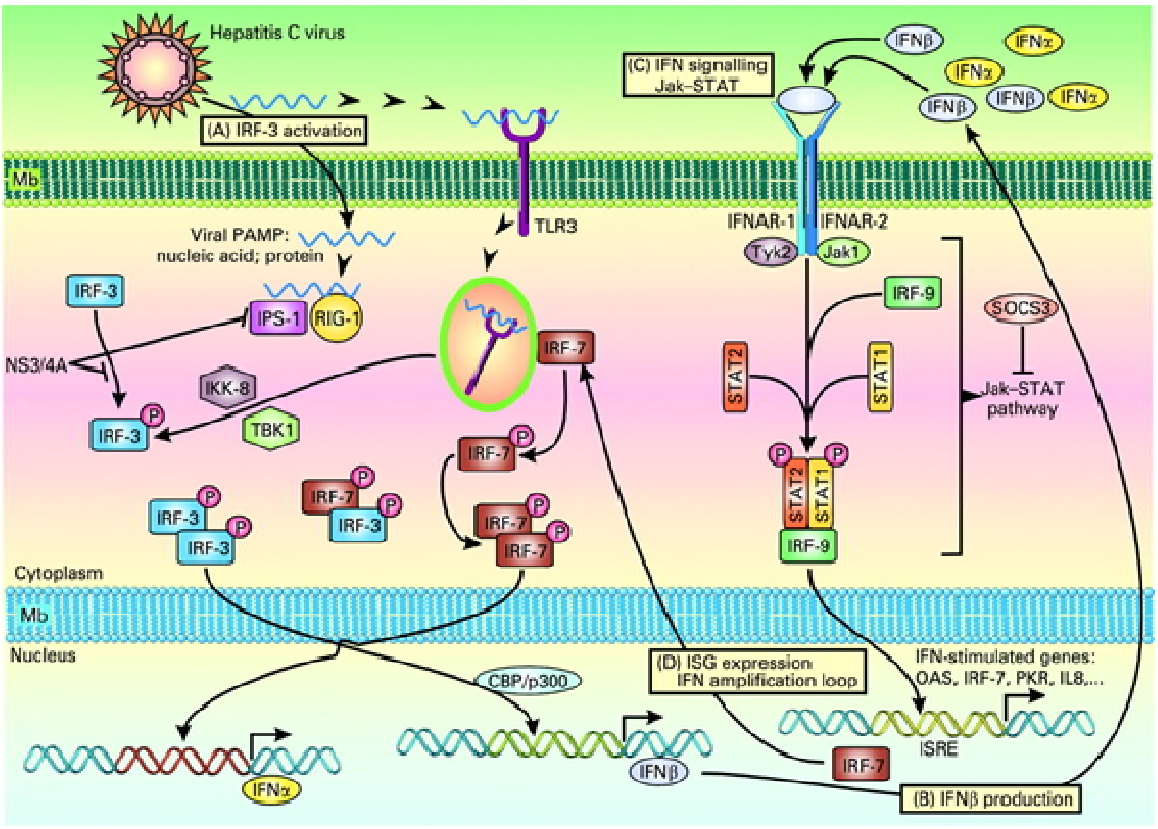


Figure 1.4: Innate immunity against HCV and the IFN pathway. (Asselah *et al.*, 2009).

1.8.1.1.2- Natural killer cells

NK cells, a heterogeneous sub-population of cytotoxic lymphocytes, represent a major component of the innate immune system. They control the infection at early stages with their non-specific cytotoxic activity (Cerwenka and Lanier, 2001; Waldhauer and Steinle, 2008).

Infected cells secrete alerting cytokines, which include IFN, Interleukins (IL): IL-12, IL-15, IL-18, IL-2, and CCL5. These cytokines activate NK cell-mediated apoptosis. NK cells recognize target cells opsonized with immunoglobulin (Ig) G complexes via their FCγ-III receptor (CD16) (Cooper *et al.*, 2001). They also recognize danger signals,

such as cells expressing abnormal or reduced levels of major histocompatibility complex (MHC) class I molecules, or pathogen-derived proteins and/or stress-induced cellular proteins (Hamerman *et al.,* 2005). Activated NK cells kill infected cells directly, using perforin, a cytolytic protein that produces pores in the infected cell membrane. These pores create a channel through which granzyme proteases pass and lyse the cell (Larkin *et al.,* 2006). NK cells also secrete a variety of cytokines, including IFN-γ, tumor necrosis factor α (TNF-α) and IL-4, which inhibit viral replication (Wang *et al.,* 2008). They also stimulate the maturation of DCs, and initiate adaptive immune responses (Ahmad and Alvarez, 2004; Bendelac *et al.,* 2007; Lanier *et al.,* 2008).

NK cells are abundant in the liver, constituting about 30% of the intra-hepatic lymphocytes, compared to 5-10% in peripheral blood (Norris *et al.,* 1998). During an acute HCV infection, NK and NKT cells are triggered by IFN α and β and play a critical role in HCV clearance (Khakoo *et al.*, 2004; Pelletier *et al.,* 2010).

1.8.1.2- Adaptive immunity

1.8.1.2.1- Humoral immune response

Humoral immunity is conferred by antibodies secreted from B-lymphocytes upon activation during an infection. Neutralizing antibodies perform a major role in the clearance of many viral infections. HCV infection induces antibodies against all viral proteins. HCV-specific antibodies appear in serum about 6-7 weeks after the initial infection. The delayed antibody response contributes to the persistence of the virus (Alter *et al.,* 1989; Rahman *et al.,* 2004). The first antibodies to be detected are those which target NS3 and core viral proteins while antibodies against NS4 and envelop proteins E1 and E2 appear later (Orland *et al.,* 2001). E2 has been a main target for neutralizing

antibodies however these antibodies failed to prevent reinfection with the same or a heterologous strain due to the high heterogeneity of E2 (Farci *et al.*, 1992; Lai *et al.*, 1994). Therefore, the significance of the role of antibodies in the establishment of protective immunity was questioned, given reports that viral clearance was detected in acute infections, even in the absence of humoral immunity (Bjoro *et al.*, 1994).

The development of HCV models that enable the study of viral neutralization provides insight into the role of HCV antibodies in viral clearance and their impact on infection outcome (Bartenschlager and Lohmann, 2001). This is demonstrated by the ability of Virus-like particles (VLP) carrying core, E1 and E2 proteins, to produce neutralizing antibodies capable of protection against HCV infection in mice (Lechmann *et al.*, 2001).

1.8.1.2.2- Cellular immune response

Cellular immunity is critical for HCV clearance. HCV proteins produce an innate immune response, which stimulates DC maturation and antigen presentation to T lymphocyte cells. DCs present antigens in association with MHC I and II, which is recognized by $CD8^+$ and $CD4^+$ T lymphocytes, respectively. DCs also secret cytokines and activate the T cells (Gonzalo *et al.*, 2001). Activation of T helper (Th) $CD4^+$ cells leads to cytokine production which differentiates $CD4^+$ cells to Th1 that activates the cytotoxic response, or Th2, which activates the humoral response. An early $CD4^+$ response is critical to HCV infection control. The Th1 phenotype in particular plays an important role in HCV resolution. Th1 helps activate and maintain cytotoxic T lymphocyte (CTL) $CD8^+$ cells. The latter mediates viral clearance either by killing the infected cells, or by producing antiviral cytokines such as IFN, or TNF-α, which inhibits

viral replication and protects neighboring cells from invasion (Cooper *et al.,* 1999; Lechner *et al.,* 2000; Woollard *et al.,* 2003). Cellular immune responses target many HCV proteins including core, E2, NS3 and NS5 (Ferrari *et al.,* 1994; Hoffmann *et al.,* 1995). Robust and sustained specific $CD8^+$ and $CD4^+$ T cells were detected in patients who could resolve the acute infection, whereas chronic infection was found to be correlated with delayed and stunted immune responses. HCV specific T cells are detected 7-10 weeks after infection, when the viral load has already reached high levels (Thimme *et al.,* 2001; 2002).

1.8.2- HCV evasion of the host immune system

Despite the efficiency of the immune system in controlling viral infections, HCV can persist in the infected liver leading to a chronic disease. Generally, a vigorous immune response leads to viral clearance, whereas weak, impaired immune responses lead to persistence of infection. Since the majority of people infected with HCV are not able to clear the infection, it is suggested that HCV must employ multiple efficient strategies to subvert the host immune response.

1.8.2.1- HCV interference with host innate immunity

Multiple mechanisms are involved in the evasion of the host innate defense system. Different viral proteins interfere with viral detection sensors and IFN induction pathways (Gale and Foy, 2005) (Figure 1.5). For instance, NS3/NS4 protease inactivates both RIG-1 and TLR-3 pathways by cleaving their adaptor molecules IPS-1 (Meylan *et al.,* 2005) and TRIF (Breiman *et al.,* 2005; Li *et al.,* 2005). In addition, HCV NS3/4A protease blocks the accumulation and nuclear translocation of phosphorylated IRF-3 and

the consequent expression of type I IFNs and IFN-stimulated genes (ISGs) (Foy *et al.,* 2003). IFN transcription is also inhibited by the interaction of NS3 and TBK1, disrupting the association between TBK1 and IRF-3 (Otsuka *et al.,* 2005).

The core protein blocks IFN signaling through the JAK/STAT pathway, by the induction of inhibitory molecules such as suppressor of cytokine signaling 3 (SOCS3) (Bode *et al.,* 2003), and phosphatase 2A (Duong *et al.,* 2004). These inhibitory molecules interfere with STAT-1 and the formation of the transcription complex, thereby inhibiting the production of ISGs.

Furthermore, the functions of several ISGs are impaired by HCV proteins. For example, PKR is inhibited by IRES (Vyas *et al.,* 2003), E2 protein (Taylor *et al.,* 1999) and NS5A (Pflugheber *et al.,* 2002). NS5A also inhibits the 2–5 OAS/RNaseL pathway (Taguchi *et al.,* 2004) and induces IL-8, which counteracts the antiviral effects of IFN (Polyak *et al.,* 2001a). Moreover, these proteins correlate with resistance to the current IFN-based therapy for HCV infection (Berg *et al.,* 2000; Witherell *et al.,* 2000).

In addition, mutations in the ribonuclease (RNase) L recognition sites in the HCV genome of certain strains allow them to escape nucleolytic cleavage, and resist IFN activity (Han and Barton, 2002).

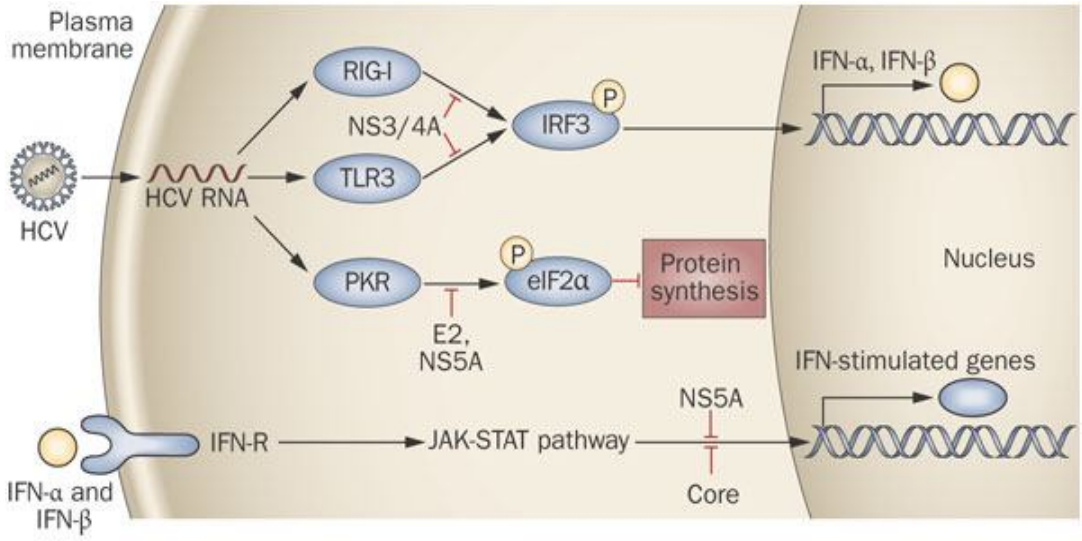


Figure 1.5: HCV evasion of the innate immune system and blockage of IFN pathways. (Sklan *et al.*, 2009).

HCV has also evolved strategies to evade the NK cell response. The envelope protein E2 has been shown to crosslink to the CD81 receptor, suggesting an inhibitory effect on the cytotoxicity and IFN-γ production by NK cells (Crotta *et al.*, 2002; Tseng and Klimpel, 2002; Yoon *et al.*, 2009). Chronically infected patients have depleted and dysfunctional NK cells (Golden-Mason and Rosen, 2006).The impaired NK cells produce inhibitory cytokines including IL-10 and transforming growth factor-β (TGF-β), which suppress the adaptive immune response (Jinushi *et al.*, 2004; Meier *et al.*, 2005). In addition, the virus renders the infected hepatocytes resistant to NK cell attacks by expressing inhibitory ligands (Herzer *et al.*, 2003; Wen *et al.*, 2008).

1.8.2.2. - HCV interference with host adaptive immunity

HCV has evolved multiple mechanisms to suppress the host adaptive immune response. HCV infection targets T cells, inhibiting their maturation, and impairing their functions involved in viral clearance. DCs are also targets for HCV evasion (Hiasa *et al.*, 1998; Sarobe *et al.*, 2002). HCV's core protein binds to the gC1q receptor on DCs, reducing the production of IL-12. This shifts the differentiation of $CD4^+$ cells towards the Th2 phenotype, which is less effective in controlling the infection (Eisen-Vandervelde *et al.*, 2004; Waggoner *et al.*, 2005). The bias in Th1 to Th2 balance is also achieved by mutation selection. A mutation in the NS3 epitope, which is known to induce Th1 immunity, results in the inactivation of $CD4^+$ (Wang and Eckels, 1999; Wang *et al.*, 2003).

Chronic HCV infection inhibits the maturation of effector $CD8^+$ T cells, suppresses their ability to produce IFN-γ, and their cytotoxic activity (Wedemeyer *et al.*, 2002; Penna *et al.*, 2007). Two mechanisms are suggested to affect $CD8^+$ cell function, either failure in primary activation or exhaustion of initially activated cells by the high viral load. One suggested mechanism for cell exhaustion is the upregulation of programmed death–1 (PD-1) receptors on T cells, which converts the activated cells to the exhausted phenotype, with an impaired ability to proliferate or release cytokines (Penna *et al.*, 2007). In addition, the $CD4^+$ $CD25^+$ regulatory T (Treg) cells increase in numbers in the liver during chronic HCV infection. Treg cells secrete IL-10, which inhibits the functions of antigen presenting cells (APCs), including antigen processing, MHC expression and IL-12 production. Treg cells also inhibit HCV-specific $CD8^+$

proliferation (Boettler *et al.*, 2005; Bolacchi *et al.*, 2006). Figure 1.6 shows the interference of HCV with host adaptive immunity.

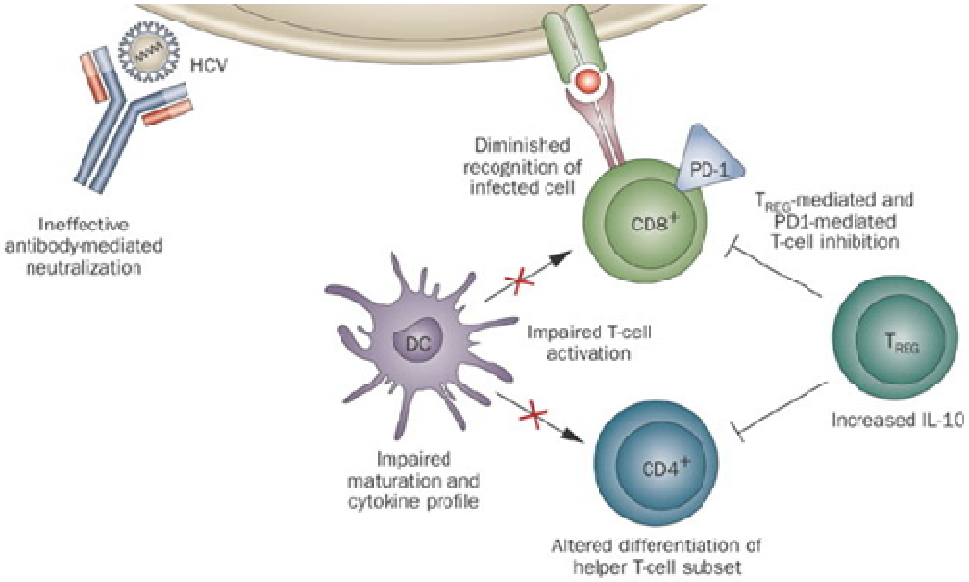


Figure 1.6: The evasion of the adaptive immune system by HCV (Sklan *et al.*, 2009).

Finally, the accumulation of mutations in the HCV genome plays a major role in immune system evasion. Mutations in immunodominant specific epitopes facilitate viral escape, due to failure in recognition by specific T or B cells or the impairment of binding of the epitope to the MHC on APCs (Bowen and Walker, 2005). Although the antibodies against the envelope proteins are more critical in viral neutralization, the high mutation rate in the HVRs of the E2 protein impairs the recognition of the neutralizing antibodies. Moreover, the heavy glycosylation of E2 suppresses the neutralizing effect of the antibodies (Farci *et al.*, 2000).

1.9- HCV Treatment

1.9.1- IFN-Ribavirin

The sequence diversity of HCV is a major hurdle to the development of an effective therapy. The current standard therapy approved by the Food and Drug Administration (FDA) is the pegylated IFN-α 2a/ 2b combined with a nucleoside analogue, ribavirin. However, sustained virological response (SVR) is only achieved in less than 40% of individuals infected with HCV genotypes 1 or 4, compared with 80% of patients infected with genotypes 2 or 3. Sequence variability within the NS5A region, particularly a consensus IFN sensitivity-determining region (ISDR) sequence and a PKR binding domain (Gale *et al.,* 1998), have been associated with IFN alpha resistance in genotypes 1 and 4 (Kurosaki *et al.,* 1997; Saiz *et al.,* 1998; Pascu *et al.,* 2004). IFN-Ribavirin therapy requires a long time period (12-72 weeks) and is associated with significant side effects, including haemolytic anemia and severe depression (Fried and Hadziyannis, 2004). In a trial to improve the current therapy, alternative modified drugs were tested. For instance, taribavirin, a prodrug of ribavirin, reduced anemia, however the therapeutic efficiency was low (Gish *et al.,* 2007). Therefore, alternative therapeutic approaches have evolved based on recent knowledge gained about the virus life cycle and the proteins involved in viral replication (Di Bisceglie *et al.,* 2002; Keeffe, 2007; Soriano *et al.,* 2008).

1.9.2- Protease and polymerase inhibitors

With increasing knowledge of the HCV life cycle, vital enzymes could be determined and targeted for inhibition. The NS3/NS4 protease and NS5B polymerase are the most attractive targets for antiviral therapy, as they are the key players in the viral life cycle.

Several compounds have already been tested against these vital proteins, and many have shown promising results (Narjes *et al.,* 2003; Llinas-Brunet *et al.,* 2004; Lamarre, *et al.,* 2003).

1.9.3- RNA intereference

RNA intereference (RNAi) is a mechanism by which long dsRNAs from an exogenous source like an invading virus or synthetic siRNA, or an endogenous source such as microRNAs (miRNAs), result in silencing of homologous gene expression.

RNAi has been observed in most organisms and is considered an antiviral mechanism in plants and invertebrates (Ding *et al.,* 2004). In mammalian cells, miRNAs have an essential role in regulating cellular gene expression (Szweykowska-Kulinska *et al.,* 2003; Yang *et al.,* 2005). Hundreds of miRNAs have been identified and 30% of proteins are regulated by miRNAs (Bartel *et al.,* 2004).

Utilization of RNAi technology seems appropriate for HCV therapy since the HCV genome is a single-stranded RNA that replicates in the cytoplasm of liver cells without integration into the host genome. Inhibition of HCV RNA translation could inhibit HCV replication, thus resolving chronic HCV infections.

The major obstacle to successful use of RNAi technology is the extreme heterogeneity of HCV (Carmichael, 2002). The high error mutation rate of HCV facilitates escape from recognition and attack by siRNAs. Therefore, for efficient silencing, siRNAs must be targeted to conserved regions of the viral genome. The 5′ NCR of the HCV genome is the most conserved among all known strains of HCV. It is also critical for protein synthesis. Sequence-specific siRNAs, targeted to the IRES at the 5' NCR, reduced viral protein synthesis and abolished intracellular replication of the viral

genomic RNA (Yokota *et al.,* 2003). The NS3 coding region has also been shown to be an effective target, due to the high degree of conservation and accessibility of the RNA-induced silencing complex (RISC). The NS3 region is vital for viral replication, as it encodes protease and helicase proteins. siRNAs against both NS3 and NS5b regions suppressed HCV replication (Takigawa *et al.,* 2004), however NS5b is more heterogeneous than NS3.

siRNAs against structural genes have also been found to be an effective therapeutic option against HCV infection. siRNAs targeting the E1 and E2 genes and core gene demonstrated dramatically reduced HCV viral copy numbers in HCV serum-infected human hepatoma (Huh-7) cells (Khaliq *et al.,* 2010; 2011; Ansar *et al.,* 2011).

Some cellular factors promote viral replication and biogenesis, making them ideal therapeutic targets. For instance, ADP-ribosylation factor 1 (Arf1) plays an important role in viral maintenance, thus downregulation of Arf1 expression by siRNA reduced both the levels of HCV RNA and the production of infectious viral particles in Huh7.5 cells (Machlin *et al.,* 2011). Taken together, these findings suggest RNAi as a potent therapy for the elimination of the HCV virus from persistently infected host cells.

1.10- Hepatitis C vaccine

Despite all the efforts towards HCV vaccine development, a vaccine is not yet available. Initially, the development of a vaccine against HCV was hampered by the lack of small animal models and a reliable cell culture system. The recent development of new HCV model systems, including HCV replicon cell lines (Lindenbach *et al.,* 2005), has provided new avenues for vaccine development, and some progress has been made. Still, the most challenging obstacle remaining is the high mutation rate of HCV, which results

in multiple viral isolates. A successful vaccine must generate vigorous broad and multi-specific $CD4^+$ and $CD8^+$ T cells, in addition to cross-genotype neutralizing antibodies (Inchauspe *et al.*, 2007; Lang and Weiner, 2008).

Since the immune response of many patients is impaired by the HCV infection, an HCV vaccine can also be used as a therapeutic tool to augment the immune system. Therapeutic HCV vaccines can be used with antiviral therapy or immunomodulators to expand their effect. Many vaccine strategies have been developed, including recombinant protein vaccines (Puig *et al.*, 2004), DNA vaccines (Forns *et al.*, 2000), and viral vector vaccines (Youn *et al.*, 2008). Prime-boost strategies were also utilized including DNA prime followed by protein boost (Rollier, *et al.*, 2004) or viral vaccine boost (Puig *et al.*, 2006; Youn *et al.*, 2005). Therapeutic vaccines were also developed using peptide vaccines (Nevens *et al.*, 2003; Klade *et al.*, 2008) and some entered phase I clinical trials (Yutani *et al.*, 2007).

Initial vaccine research was based on recombinant proteins and showed promising results. For instance, HCV E1/E2 glycoprotein emulsified with MF59 (sub-micron oil-in-water emulsion of a squalene adjuvant) induced a potent humoral immunity as evidenced by a strong $CD4^+$ T cell response and specific neutralizing antibodies (O'Hagan *et al.*, 2004). However E1/E2 protein vaccine was able to induce a strong cytotoxic $CD8^+$ T cell response when followed by a boost dose of defective alphaviral particles with RNA encoding for HCV E1/E2/NS3-5 (Lin *et al.*, 2008). Several reports show that cellular immunity is critical for HCV clearance. This suggests that the DNA and viral vaccines are more effective as they induce both humoral and cellular immunity (Lechner *et al.*, 2000; Lauer and Walker, 2001). DNA vaccine technology provides a promising approach

that has several advantages over conventional vaccines and extensive research has been done to bring DNA vaccines to clinical use. Moreover, DNA vaccines have proven safe and immunogenic in several clinical trials. In addition, recombinant viral vaccine is another gene-based vaccine approach that combines the advantages of a DNA vaccine with a potent viral delivery system. Recombinant viral vaccines have also been tested in clinical trials as the primary vaccine or as a booster for a DNA vaccine.

1.10.1- DNA Vaccines against HCV

DNA vaccines have been used extensively in HCV vaccine research using different HCV genes. E1/E2 plasmid vaccines also triggered a Th1-like cellular immune response and increased IFN-γ secretion (Zhu *et al.,* 2004). E2-encoding plasmid induced neutralizing antibodies (Owsianka *et al.,* 2008) and a potent Th1 cellular response (Nam *et al.,* 2006). Antigen localization has also been found to affect the outcome of vaccine administration. For instance, a secreted form of E2 enhanced the humoral immune response due to the availability of the protein to the immune pathways (Ma *et al.,* 2002; Li *et al.,* 2006). Optimizing cytosine-phosphate-guanine (CpG) motifs on the plasmid backbone has also been found to induce a significant increase in E2 antibody titers (Ma *et al.,* 2002). Although the E2 antigen has been used due to its greater immunogenicity, E1 antibodies showed broader reactivity. A multi-genotype vaccine encoding different genotypes of E1 was found to successfully induce cross-reactive immune responses both on the humoral and cellular levels (Encke *et al.,* 2007).

The highly conserved core antigen has also been considered as candidate immune target for the induction of a cross-reactive CTL response. Core encoded plasmid vaccines induced long-lasting humoral and cellular immune responses in mice (Lagging *et al.,*

1995; Arichi *et al.*, 2000; Duenas-Carrera *et al.*, 2000) allowing them to enter clinical trials as therapeutic DNA vaccines in HCV-chronically infected patients (Alvarez-Lajonchere *et al.*, 2009). Administration of cytokines modulates the immune response. For instance, the CTL response elicited by the HCV core was enhanced when co-administered with IL-2, and suppressed by IL-4 (Geissler *et al.*, 1997). Multiple antigens were evaluated in a DNA vaccine encoding the structural genes E1/E2/core, inducing a potent CTL response (Nishimura *et al.*, 1999). However, core proteins were shown to interfere with the host immune response (Yoshida *et al.*, 2001; Zhu *et al.*, 2010) and to cause cellular transformation in mice (Moriya *et al.*, 1998; Lerat *et al.*, 2002).

The NS3 protease/helicase is another attractive target. It is a large protein that potentially contains numerous epitopes. Moreover, NS3 and core antigens are the most conserved regions in the HCV genome. NS3 was extensively explored in DNA vaccines in small animals using various approaches. Plasmids encoding a codon-optimized NS3, with its cofactor NS4A, greatly enhanced CTL-mediated immunogenicity by increasing expression and intracellular stability of NS3 (Frelin *et al.*, 2004; Ahlen *et al.*, 2005). NS3/NS4A delivered by gene gun biased the balance towards a Th2 immune response (Frelin *et al.*, 2003). Multigene vaccines encoding NS3, core and different genotypes of E1, have also been found to induce high cross reactive E1 antibody titers as well as high levels of specific NS3 and core CTL responses. These multigene vaccines also shift the immune response towards Th1, and induce high levels of IFN, and show robust prophylactic and therapeutic effects in mice (Zeng *et al.*, 2009).

1.10.2- Recombinant viral vaccines against HCV

Viral vectors with low virulence that express heterologous genes in the host were successfully used for vaccination, thus providing several advantages. The viral vector vaccines have the same advantages as DNA vaccines in intracellular production of the antigen, thus activating both cytotoxic T cells and the antibody response. However, they are preferred for their ability to infect various types of cells, including APCs (Miyazawa *et al.,* 1999; Brown *et al.,* 2003), and efficiently use the host cell machinery to induce high and prolonged foreign gene expression. Moreover, viral pathogen-associated molecular patterns (PAMPs) stimulate the innate immune response and work as adjuvants (Molinier-Frenkel *et al.,* 2002).

Several viral vectors have been used in HCV vaccination, including adenovirus, vaccinia virus and canarypox virus. Adenoviruses (Ad) were shown to be a highly efficient tool for vaccine delivery, due to their broad tropism. Ad vectors induce a $CD8^+$ cytotoxic response, which is critical for HCV clearance. Immunization of mice with 10^9 plaque forming units (PFU) of recombinant adenovirus expressing NS3, induced NS3-specific humoral and cellular responses, as well as $CD8^+$-mediated protection against infection with the vaccinia virus expressing HCV polyproteins (Arribillaga *et al.,* 2002). Co-administration of rAdNS3 vaccine with cytokines, like IL-12, or fusion of NS3 to stimulatory molecules, like the MHC class II chaperone protein invariant chain, results in a rapid, potent and prolonged cellular immune response in mice with production of IFN-γ, TNF-α and IL-2. This vaccine also protected mice from infection with vaccinia virus encoding heterologous HCV-NS3 (Mikkelsen *et al.,* 2011).

One limitation to the Ad vaccine is the pre-existence of neutralizing antibodies against the commonly used Ad5 serotype in the majority of the human population. To overcome this problem, two studies looked at using alternative adenovirus serotypes, involving the immunization of rhesus macaques with HCV- NS genes expressed by the less prevalent serotype Ad6 (Capone *et al.,* 2006), or with two heterologous Ad vectors in a prime-boost strategy (Fattori *et al.,* 2006). These strategies induced a robust, cross reactive cellular-mediated immune response against HCV antigens, lasting for 2 years, indicating the maintenance of memory cells.

1.10.3- HCV Preclinical Vaccine Trials

Encouraged by the positive results obtained in mouse studies, several vaccines were further assessed in chimpanzees, the only animal model susceptible to HCV infection. The initial HCV vaccine candidate, recombinant envelope glycoproteins (gpE1/E2) of HCV combined with an oil/water adjuvant, induced high levels of neutralizing antibodies and a Th1 cell response in chimpanzees. The incidence of chronic infection in immunized chimpanzees after their trial with the heterologous virus was substantially lower than in the unimmunized animals (Choo *et al.,* 1994; Houghton *et al.,* 1997). These promising results allowed this vaccine to proceed to a Phase 1 clinical trial.

DNA-based vaccines encoding HCV envelope antigens E1 and E2 were also used in a prophylactic vaccine, inducing antibodies that could prevent persistence of the infection in chimpanzees injected with the homologous strain (Forns *et al.,* 2000). Another approach is based on a DNA vaccine prime, encoding structural genes core/E1/E2 and nonstructural genes NS3/4/5 followed by a booster Ad vector expressing

NS3-5. This strategy induced high levels of specific humoral and cellular responses as well as a significant reduction in viral load after being challenged with the heterologous virus. Interestingly, the high E2 antibody response correlated with sterilizing immunity in one of the animals (Youn *et al.,* 2005). Using NS genes NS3-NS5 in an adenovirus-based vaccine followed by electroporation of a plasmid encoding the same NS genes produced a high CTL cellular response, which could modulate the acute infection of heterologous virus-challenged animals. However, sterilizing immunity was not achieved due to the absence of neutralizing antibodies (Folgori *et al.*, 2006).

A new approach, based on non-infectious VLP, has demonstrated promising results. HCV VLPs were developed to mimic the natural virion, and to display the structural proteins on the surface, giving them the ability to infect host hepatocytes and to induce humoral and cellular immunity similar to the natural infection. HCV VLPs were tested in chimpanzees and shown to control viremia in all animals challenged with a homologous strain of HCV (Elmowalid *et al.*, 2007).

Rollier *et al.,* (2007) tested a heterologous prime boost strategy using core/ E1/E2 and NS3 encoded by plasmids, as well as a modified vaccinia ankara virus (MVA). Challenge with the same HCV strain showed potent humoral and cellular responses that reduced viremia in acute infection, but failed to protect the animals from chronic infection.

Chimpanzees immunized with replicating vaccinia virus encoding all the 5' end of the HCV ORF, encompassing the capsid up until the NS3 gene, displayed a strong, long-lasting NS3-specific cellular and humoral immune response. Challenging the animals with the same strain led to viral clearance, whereas the infection persisted when the

animals were injected with a pool of six heterologous strains, but at a very low level. This suggests that vaccinia virus is a promising vaccine candidate (Youn *et al.,* 2008).

1.10.4- HCV vaccine clinical trials

Promising preclinical results propelled HCV vaccines into clinical trials. Several vaccine approaches were tested in mice and chimpanzees, but only a few of them progressed to clinical trials. In this section, only the trials involving DNA-based vaccines, including plasmid or viral vectors will be emphasized.

CICGB-230 was the first DNA-based vaccine to reach clinical trial for HCV infections. This Phase I trial took place in Cuba. The vaccine consisted of a combination of a plasmid expressing the HCV structural antigens (core/E1/E2), and core recombinant proteins. CICGB-230 was evaluated by intramuscular (i.m.) injection in 15 patients chronically infected with the HCV genotype-1, who did not respond to the PEG-IFN/ribavirin therapy. The course of immunization lasted six months. After immune system analysis, the immunization was shown to induce neutralizing antibodies and cellular immunity. Although the viral load did not change, the patients showed stabilization or improvement in liver histology, and reduced fibrosis (Alvarez-Lajonchere *et al.,* 2009).

ChronVac-C, Tripep is another DNA vaccine evaluated in a clinical Phase I/IIa trial in Sweden. The vaccine was based on a plasmid encoding NS3/NS4A under the control of CMV ie promoter. The codons of NS3 were optimized to allow for enhanced expression in human cells. This vaccine was evaluated in 12 HCV patients infected with HCV genotype-1 who were not subjected to treatment, yet had a low viral load. The vaccine was administrated by i.m. injection, followed by *in vivo* electroporation, to

enhance the antigen uptake. The preliminary results demonstrated the development of T-cell response that was correlated to a reduction in the viral load in patients who received a higher dose. Moreover, the vaccine was proven to be safe (Sallberg *et al.*, 2009).

Ad vectors were also evaluated as a prophylactic vaccine in a Phase I trial. The vaccine was based on a replication defective adenovirus expressing NS3-NS5, with an inactivated HCV polymerase, to ensure safety. Healthy volunteers were immunized with two heterologous Ad vectors from rare serotypes, human Ad6 and the simian AdCh3, to overcome the problem of pre-existing anti-Ad antibodies. The prime-boost regime was shown to be highly immunogenic, with limited side effects. The Ad vaccine induced potent multi-specific $CD4^+$ and $CD8^+$ immune responses, as well as long lived memory T cells (Barnes *et al.*, 2009; 2012).

THE PROJECT GOALS

As seen in the review, a vaccine against HCV remains elusive partly due to virus diversity and partly due to the inefficiency of the current vaccine approaches explored to date. Although a great deal of research was done and promising results were obtained, no HCV vaccine is currently available. The goal of this project is to evaluate three different approaches for vaccination and treament against HCV as follows:

1- Evaluate two Ad promoters MLP, and AdE1A, compared to CMV ie promoter, and study their effect on the efficacy of DNA vaccines encoding the HCV genes E1 and NS3.

2- Evaluate an adenovirus vaccine encoding HCV NS3.

3- Evaluate siRNA sequences against the NS3 region and study their efficiency for the inhibition of NS3.

4- In addition, miRNAs are evaluated as novel parameters to measure the immune response elicited by the immunization.

Chapter 2

PROMOTER EFFECT ON DNA VACCINE EFFICACY

2.1- Introduction

DNA vaccination is a promising approach that depends on the delivery and expression of the antigen in the host cell. Thus DNA vaccines induce both humoral and cellular immune responses. DNA vaccination is an ideal approach for protection against HCV, particularly given the difficulty in growing HCV in cell culture for a live vaccine. In addition, evidence has shown that cellular immunity plays a critical role in HCV clearance (Thimme *et al*., 2002; 2008; Rehermann *et al*., 2005) and neutralizing antibodies are of vital importance in preventing HCV infection (Cooper *et al*., 1999). Viral genetic diversity represents a major challenge to vaccine development (Timm *et al*., 2007). Therefore, choosing the correct antigen is critical in order to design an effective DNA vaccine. Antigen targets should be highly conserved and immunogenic. The NS3 gene is the most conserved region in the viral genome and induces a strong immune response. E1 is a good target for neutralizing antibodies, due to its limited variability and inter-genotype cross-reactivity compared to E2 (Zibert *et al*., 1997). Furthermore, the function of E1 was found to be impaired in chronic HCV infection, suggesting a role in viral clearance (Depraetere and Leroux-Roels, 1999). Therefore, NS3 and E1 are ideal candidates for a prophylactic and therapeutic vaccine capable of eliciting a cellular immune response that prevents the infection and eliminates the infected cells (Ward *et al*., 2002; Shoukry *et al*., 2004).

DNA vaccines were evaluated against several viruses and successfully produced protective immunity in small animals but they showed lower efficacy in large animals and humans. Subsequently, a number of strategies were employed to enhance the efficacy of DNA vaccines. These approaches include improving antigen expression and stability, addition of adjuvants and immune modulators, using different delivery methods and routes, codon optimization and heterogeneous boosting (reviewed in Manoj *et al.*, 2004). Antigen expression level is an important factor affecting the immune response triggered by DNA vaccines that has yet to be optimized. Antigen levels are influenced by promoters. Most studies of DNA vaccines have utilized standard promoters. The most widely used is the immediate early cytomegalovirus promoter (CMV ie) due to its strong constitutive activity in most cell types (Boshart *et al.*, 1985; Furth *et al.*, 1991; Mao *et al.*, 2008). Therefore, the effect of different promoters on the efficiency of DNA vaccines against HCV was evaluated in this thesis. Two different Ad promoters were tested, the weak immediate early E1A promoter (E1AP) and the strong major late promoter (MLP), and these results were compared to the standard CMV ie promoter.

E1Ap is a weak immediate early promoter responsible for transcription of the early region E1A of Ad. The transcription of the E1A region is initiated immediately after infection, since it depends on cellular transcription factors (Nevins *et al.*, 1979). The maximal and early activity of the E1A promoter is due to the interaction of cellular transcription factors E2F (Kovesdi *et al.*, 1987) and EF-1A (Bruder and Hearing, 1989) with the enhancer elements of the promoter.

The MLP drives transcription of the major transcriptional unit of Ad which encodes the structural proteins required for virion assembly. The activity of MLP is weak

during the early phase of adenovirus infection, and greatly increases during the late infection, following viral DNA replication (Shaw and Ziff, 1980).

In this study, plasmids were constructed encoding HCV antigens NS3 or E1, under the control of the three promoters, CMV ie, MLP and E1AP. The effects of promoters on antigen expression as well as the antibody level were evaluated. In addition, microRNAs that are involved in immune response regulation were evaluated as indicators of the immune response.

2.2- Methods

2.2.1- Amplification of HCV genes NS3 and E1

HCV cDNAs, NS3 and E1, were obtained by RT-PCR amplification from the total RNA of the Huh7.5-Con1/FL-Neo cell line harboring the HCV viral genome of 1b genotype (a kind gift from Dr. Rice, Rockefeller University, USA). Two hundred nanograms of total RNA was used in a 20 μL reverse transcription (RT) reaction using NS3 or E1 specific RT primers. Four microliters of the cDNA was used in a PCR reaction with 0.12 μL of forward and reverse primers (50 μM stock), 0.2 μL deoxynucleotide triphosphates (dNTPs) (10 mM). The reaction program started with initial denaturation for 5 min at 95^{o} C. This was followed by 35 cycles, each consisting of denaturation at 95^{o} C for 30 sec, annealing for 30 sec at the appropriate temperature, and extension at 72^{o} C for 1-2 min (1 min for a 500 bp fragment) using *Pfu* polymerase (Fermentas). At the end of the reaction, the final extension cycle was performed at 72º C for 10 min. The primers were designed using the sequence of the HCV-1b strain from the nucleotide database at http://www.ncbi.nlm.nih.gov/nuccore (GenBank: AJ238799.1) and ordered from Sigma Genosys. Each fragment was designed to have an *Nco*I and *Bam*HI restriction site, as well as start and stop codons in the forward and reverse primer, respectively, to facilitate cloning and expression. NS3 is CG rich, with long CG stretches over the entire gene. It also has a strong secondary structure. Therefore, 5% dimethyl sulfoxide (DMSO) (Roche Applied Science) was added to the PCR reaction to inhibit the secondary structure. In addition NS3 was amplified in 2 parts that were ligated at

overlapping region after digestion with *Avr*II. Figure 2.1 shows the HCV genome. All primers used for this chapter are listed in Table 2.1**.**

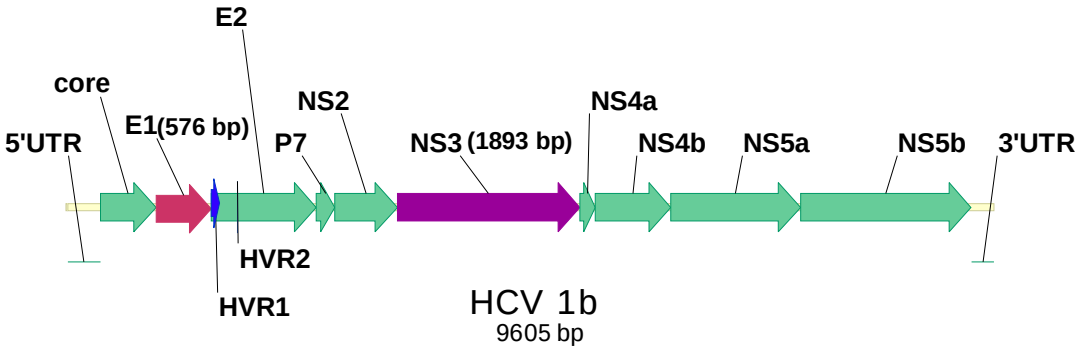


Figure 2.1: Schematic diagram of the HCV genome showing the location and sizes of E1 and NS3. Truncated E1 and complete NS3 were amplified by RT-PCR from the total RNA of HCV permissive cells (Con1/FL-Neo).

2.2.2- Plasmid construction

NS3 and E1 cDNAs were separately cloned under 3 different promoters, CMV ie, MLP, and E1A. The E1Ap plasmid was constructed by cloning adenovirus E1A promoter and poly A fragments in pUC19. pMTGA and pCG (Norgen Biotek corp.) were plasmids containing the MLP and CMV ie promoter respectively. The cDNAs were inserted in *Nco*I and *Bam*HI restriction sites under E1A and MLP and in *Age*I and *Not*I under the CMV ie promoter. All plasmids were confirmed by restriction digestion and sequencing. Small-scale plasmid DNA isolation was carried out using the Plasmid DNA MiniPrep Kit (Norgen Biotek Corp.) according to the manufacturer's instructions. Large scale endotoxin free plasmid DNA was prepared using Endotoxin Free Plasmid DNA MaxiPrep Kit (Norgen Biotek Corp.) according to the manufacturer's instructions. The plasmid DNA was concentrated by ethanol precipitation and resuspended in endotoxin free phosphate buffer saline (PBS) to a final concentration of 1 mg/ mL.

2.2.3- Cell culture

2.2.3.1- Cells and maintenance

The cells used for *in vitro* analysis throughout the course of this work include Con1/FL-Neo cells, and HEK 293 cells.

Con1/FL-Neo, a sub-line of human hepatoma cells containing the HCV strain 1b subgenomic RNA replicon, was a kind gift from Dr. Rice (Rockefeller University, USA). Cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen Corp., Gibco®), supplemented with 10% fetal bovine serum (FBS) (Hyclone), non-essential amino acids (Invitrogen Corp., Gibco®), 750 mL G418 sulphate (CalBiochem), 100 units/mL penicillin, and 100 µg/mL streptomycin (Invitrogen Corp., Gibco®), at 37° C in 5% CO_2. Cells were passaged every 3 days, at a split ratio of 1:2 after treatment with 0.05% trypsin and 0.02% EDTA (Blight, *et al.*, 2002).

HEK 293 cells (Graham *et al*., 1977; Microbix Inc., ATCC CRL-1573) were maintained in minimum essential medium (MEM) (Invitrogen) supplemented with 10% (v/v) FBS (Hyclone), 1% (v/v) Antibiotic-Antimycotic (10,000 units/mL penicillin G sodium, 10,000 µg/mL streptomycin sulfate, and 25 µg/mL amphotericin B; Invitrogen Corp., Gibco®), 1% GLUTAMAX^{TM}-1 (Invitrogen Corp., Gibco®), 3% (v/v) sodium bicarbonate (Invitrogen Corp., Gibco®), at 37° C in 5% CO_2. HEK 293 cells were passaged every 3 days at a split ratio of 1:2 after treatment with saline citrate (15 mM sodium citrate, 135 mM potassium chloride).

2.2.3.2- Cell cryopreservation and thawing

For keeping cell stock, cells were frozen when they grew to the late log phase (when the cell monolayer was 90% confluent). The cells were detached, counted and

centrifuged at 200 x g for 5 min at 4° C and then resuspended in FBS containing 10% DMSO at a concentration of 2 x 10^6 cells/mL. The cell suspension was aliquoted into cryovials (Sarstedt) and slowly frozen by wrapping them in insulating tissue towel, followed by storage at -70° C. When thawing frozen cells, the cells were removed quickly from storage and thawed in a 37° C water bath. The cells were diluted in 10 mL of growth medium, and centrifuged at 200 x g for 5 min. The cell pellet was resuspended in 20 mL of complete growth medium, plated on a 100 mm tissue culture plate and incubated at 37° C with 5% CO_2.

2.2.3.3- Mammalian cell counting

The cells were lifted and resuspended in the proper medium. The cell suspension was counted using a hemocytometer (Improved Neubauer, Hausser Scientific) under an inverted light microscope, and the number of total cells in the original suspension was calculated according to the equation:

Cell concentration/mL = Total cell count in the 4 large corner squares x 2500 x dilution factor.

2.2.3.4- Mammalian cell transfection

HEK 293 cells were transfected with different plasmids encoding E1 and NS3 to evaluate the promoter effect on gene expression. When the cells were 80-90 % confluent, they were transiently transfected with endotoxin free plasmid using lipofectamine 2000 (Invitrogen Corp., Gibco®) according to the manufacturer's instructions. Briefly, the cells were plated in antibiotic-free medium to avoid cytotoxicity. For a 6-well plate, 500 µL transfection mix was used per well. Five microliters of lipofectamine was diluted in 250 µL Opti-MEM I Reduced Serum Medium (Invitrogen Corp., Gibco®) and incubated

for 5 min at room temperature. It was then mixed with 250 µL of Opti-MEM I Medium containing 5 µg of the plasmid DNA. The mix was incubated for 20 min before being added dropwise to the well. The cells were incubated for 6 h followed by changing of the medium with the complete medium containing an antibiotic.

2.2.4- *In vitro* evaluation of transcriptional activity of different promoters

2.2.4.1- RNA isolation

Total RNA was isolated using the Total RNA Purification Kit (Norgen Biotek Corp.) according to the manufacturer's instructions.

2.2.4.2- RNA quantification

RNA was quantified using a spectrophotometer after dilution with water. The absorbencies were measured at wavelengths 260 nm (A260) and 280 nm (A280) and the RNA concentration was then calculated according to the following formula (Sambrook *et al.*, 1989): RNA concentration (µg/ µL) = (A260) × (dilution factor) × (40 ng/ µL)/1000. The ratio A260/A280 was used to assess RNA purity. An A_{260}/A_{280} ratio of 1.8- 2.1 is indicative of highly purified RNA.

2.2.4.3- Formaldehyde agarose gel electrophoresis

The quality of RNA samples was also assessed by electrophoresis on a formaldehyde agarose gel, prepared according to Sambrook *et al.* (1989). The gel was viewed under UV light and images were captured with AlphaImager 2200 (Alpha Innotech).

2.2.4.4- DNAse I treatment and cleaning of RNA

The DNA contamination in the isolated RNA samples was degraded with TURBO deoxyribonuclease (DNaseI) (Ambion). Fifty microlitres of each RNA sample was

digested in a 100 µL reaction mixture containing the provided buffer and 4 units of TURBO DNaseI, then incubated at 37° C for 30 min. RNA samples were cleaned after DNase I treatment with the RNA Clean Up and Concentration Kit (Norgen Biotek Corp.), according to the manufacturer's instructions.

2.2.4.5- Quantitative RT-PCR:

The quantitative (q) RT-PCR was performed to quantify the transcription levels of NS3 and E1 under different promoters. The RT reaction was performed using 200 ng of RNA combined with 0.5 µL RT-specific primer (50 mM stock) in 5 µL final volume. This mixture was incubated for 5 min at 70° C, and then cooled at 4° C. Fifteen microliters of the RT reaction solution was added to the mixture. The RT reaction solution contained 4 µL of 5X First Strand Buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl and 15 mM MgCl2), 2 µL of 0.1 M Dithiothreitol (DTT), 1 µL of 10 mM dNTPs, 0.1 µL Superscript III reverse transcriptase (Invitrogen) and 7.9 µL RNase/DNase-free water. The reaction was incubated at 25° C for 5 min, followed by 90 min at 50° C. The reaction was inactivated by heating at 70° C for 15 min and finally held at 4° C.

The q-PCR reaction was performed in a Bio-Rad iCycler thermal cycler. Briefly, for 20 µL total reaction volume, 10 µL of 2 x CYBR GREEN master mix (BioRad) was mixed with 1.2 µL of forward and reverse primers (5mM stock) and 4 µL of cDNA template. The q-PCR reaction starts with 15 min at 95^{o} C followed by 40 cycles of three steps: 95^{o} C for 15 sec, 60^{o} C for 30 sec and 72^{o} C for 30 sec. The reaction was incubated for 1 min at 57^{o} C before starting a melting curve analysis by 0.5^{o} C increments every 10 sec over 80 rounds. Gene transcription levels were determined using absolute quantification. A standard curve was prepared from NS3 or E1 plasmid using known

concentrations. The concentration of unknown samples was then determined by simple interpolation of cycle threshold (Ct) values into the standard curve.

Table 2.1: List of the primers used for construction and q-PCR

Replicon	Primer	Sequence	amplicon size
E1A promoter	E1P3Fw	5' ATATATCATATGGACGGATGTGGCAAAAGTGA 3'	435 bp
	E1P2 Rv	5' AAGAATTCCCATGGTTTCAGTCCCGGTGTCGGAG 3'	
E1A prolyA	PolyA Fw	5' CTGCAGGGTGTAAACCTGTGATTGC 3'	180 bp
	polyA Rv	5' AAGCTTAGGTCAGATGTAACCAAGAT 3'	
HCV-E1 for cloning	conE1-RT	5' GTCAACGCCGGCAAAGAGTAG 3'	
	conE1RS-Fw	5' GCATGCCATGGGATATGAAGTGCGCAACGTATC 3'	488 bp
	conE1tRS-RV	5'CGATGATTGCGGCCGCTTAGATCCGGAGTAACTGCGATA3'	
HCV-E1 for q-PCR	conE1-Fw	5' ATGTATGAAGTGCGCAACGTATCC 3'	457 bp
	conE1tr-RV	5' TTAGATCCGGAGTAACTGCGATACC 3'	
HCV-NS3 for cloning	RT-NS3con1	5' GCTCGTGACGACCTCCAG 3'	
	HCNS3-1Fw	5' CACCCTGCCATGGCGCCTATTACGGCCTACTC 3'	780 bp
	HCNS3-1Rv	5' CAATTATTGCGGCCGCTAAGGGTCGATACCATGTGCC 3'	
	HCNS3-2Fw	5' GCATGCCATGGCAGCCCAAGGGTATAAGGT 3'	1270 bp
	HCVNS3-Rv	5' CGATGATTGCGGCCGCTACGTGACGACCTCCAGGT 3'	
HCV-NS3 for q-PCR	NS31Fw	5' CCAAATGTACACCAATGTGGACC 3'	283 bp
	NS31Rv	5' TGGTTTCCATAGACTCGACGG 3'	
	NS32Fw	5' GCTGTGCTTGGTACGAGCTC 3'	396 bp
	NS32Rv	5' AGCCGTATGAGACACTTCCAC 3'	

2.2.5- Expression of E1 and NS3 proteins

2.2.5.1- Protein expression

NS3 and E1 were expressed in *E.coli* expression strain BL21(DE3)pLysS (Novagen). E1 and NS3 were cloned under the T7 promoter in 2 plasmids, pT7E1 and pT7NS3. The plasmid encoding the gene of interest was transformed in pLysS bacteria. One colony was suspended in 2 mL Luria-Bertani (LB) media, containing 1 μg/ mL ampicillin and 34 μg/ mL chloramphenicol, and cultured overnight in a shaking incubator at 37^{o} C. The culture was inoculated in 50 mL LB, and grown until the OD_{600} reached ~0.6. The culture was then centrifuged for 10 min at 4000 rpm. The pellet was suspended in 1L LB, and was then grown once again. When the OD_{600} reached ~0.6, the culture was induced by 0.5 mL of 50 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and incubated for an additional 3 h in a shaking incubator at 37° C.

2.2.5.2- Protein isolation from inclusion bodies

The expressed protein was isolated from the culture of the pLysS strain after IPTG induction. The isolation was carried out using the ProteoSpin Inclusion Body Protein Isolation Maxi Kit (Norgen Biotek Corp) according to the manufacturer's instructions.

2.2.5.3- Protein gel electrophoresis

Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). ProteoLadder 125 or 150 (Norgen Biotek. Corp) was used to determine the protein molecular weight. The polyacrylamide stock solution was prepared to an acrylamide concentration of 30% w/v at a ratio of 29 acrylamide: 1 bisacrylamide (Sambrook *et al.*, 1989). Prior to gel loading, the protein samples were

prepared by heating for 10 min at 90°C in a protein loading dye (125 mM Tris-HCL pH 6.8, 10% 2-mercaptoethanol, 10% SDS, 10% glycerol and 1% bromophenol blue). The gel was run at 15 V/cm in Tris-glycine electrophoretic buffer (25mM Tris, 25mM glycine [pH 8.3] and 0.1% SDS). The gel was stained with Coomasie Brilliant Blue R250 (0.25% [w/v] dissolved in 45% methanol, and 10% glacial acetic acid) for 2 h. After rinsing, the gel was incubated in destaining solution (45% methanol, 10% glacial acetic acid) overnight with agitation.

2.2.5.4- Protein quantification (Bradford assay)

The BioRad colorimetric reagent, based on the Bradford assay (Bradford, 1976), was used to determine the concentration of protein extracts. In this assay, a set of standards was created from bovine serum albumin (BSA) (NEB) with different concentrations. T he standards and the unknown protein sample were mixed with the Bradford reagent, and the absorbance of the resultant blue color was measured at 595 nm following a short incubation at room-temperature. A standard curve was constructed using the standard OD values, and the concentration of the protein samples was determined based on the equation of the line, generated from the standard curve.

2.2.6- Animals and immunization protocol

Female BALB/c mice, 6-8 weeks old, (18-20 g) were purchased from Charles River Breeding Laboratories, and were housed at the animal care facilities at Brock University. All animal work was approved by the Brock University Animal Care and Use Committee (ACC) and done according to the Canadian Council on Animal Care (CCAC) guidelines required for experimentation with animals. Three animals were used in each group. Immunization with the DNA vaccines was accomplished by injecting 50 µg of

plasmid DNA (in 100 µL final volume of endotoxin free PBS) into the *tibialis anterior* (TA) muscle, divided on the left and right muscles. Each animal received three i.m. injections, at two-week intervals. The control animals were injected with 100 µL PBS. Mice were anaesthetized by inhalation of 5% isoflurane with oxygen prior to injection.

2.2.7- Blood collection and serum preparation

Blood samples were collected by heart puncture 2 wks after the last immunization. The blood was incubated in an upright position at room temperature for 30-45 min to allow for clotting, and then centrifuged at 7000 x g for 15 min at 4^{o} C. The serum (supernatant) was collected and small aliquots were stored at -80^{o} C.

2.2.8- Immunological analysis

2.2.8.1- Enzyme-linked immunosorbent assay (ELISA)

An ELISA test was used to monitor the levels of NS3 and E1 specific antibodies in the sera collected from the immunized animals (Zeng *et al.,* 2009). ELISA Immulon 4HBX plates (96-well) (VWR International, LLC) were coated with recombinant NS3 or E1 protein (expressed in *E.coli*) at 0.1µg/well in a carbonate buffer (50 mM $NaHCO_3$ buffer, pH 9.6) overnight at 4^{o} C. The plate was washed 5 times with wash Phosphate Buffered Saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4 and1.47 mM KH2PO4, pH of 7.4) + 0.1% Tween 20. The active sites on the plates were blocked with wash solution containing 2% BSA for 2 h at room temperature to block non-specific antibody binding. Serial dilutions of the sera were incubated with the protein for 2 h at room temperature on an orbital shaker. To determine the IgG levels, 100 µL of Peroxidase-conjugate goat anti-mouse IgG antibody (Sigma) at a dilution of 1:5000 was added to each well. The reactions were developed with o-phenylenediamine

dihydrochloride (OPD) substrate (Sigma) at 200 µL/well followed by 30 min incubation at room temperature. The optical density (OD) was measured at 450 nm by the Thermo Scientific® ELISA reader. OD values had to be at least twice the values of the control (non-immunized) animals to be considered positive (Zhu *et al.*, 2004).

2.2.8.2- Protein immunoblot (western blot)

Western blot assays were performed to confirm the specificity of NS3 and E1 antibodies in the serum of the immunized mice (Brett *et al.*, 2004). Briefly, the proteins were separated on a 10% SDS-polyacrylamide gel followed by electrophoretic transfer to a polyvinylidene difluoride (PVDF) membrane (Pall Sciences). The membrane was soaked in methanol, followed by soaking in 1X Blotting Buffer (25 mM Tris, 192 mM glycine, 10% methanol) (Towbin *et al.*, 1979) along with the blotting paper, and the gel containing the protein. The transfer unit was then assembled according to the manufacturer's instructions, and the proteins were transferred to the PVDF membrane at 40 mA per gel for 1 h. The PVDF was then blocked overnight using 5% powdered milk in TBST buffer (50 mM Tris base, 150 mM NaCl, 0.05% Tween 20, pH 7.6), washed 3 times with TBST, and incubated for 2 h with pooled serum from an animal group (diluted to 1:500 in TBST with 0.5% milk). After washing, the PVDF membrane was hybridized with 1/2500 dilution of horse radish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma). The membrane was washed and developed by chemiluminescent detection using the Pierce CN/DAB Kit (Thermo Fisher Scientific).

2.2.8.3- Quantification of serum miRNA levels

Total RNA was isolated using the Total RNA Purification Kit (Norgen Biotek Corp.) The expression levels of different miRNAs were quantified by qRT-PCR using specific primers. The RT reaction was performed using stem–loop RT primers specific for each miRNA. The q-PCR reaction was carried out using specific forward primers for each miRNA and one universal reverse primer (miRNA-RV) complementary to the stem loop (table 2.2). The Ct values were normalized to the 5SrRNA gene. The fold change values were calculated with the ΔΔCt method. The expression levels of miRNAs were indicated as fold-difference in expression compared to the non-immunized animals.

Table 2.2: The primers used for miRNA quantification

PCR product	Primer	Sequence
	miRNA-RV	5’ GTGCAGGGTCCGAGGT 3’
mir-181	mir-181-RT	5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTCAC 3’
	mir-181-Fw	5’ AACATTCAACGCTGTCGGT 3’
mir-155	mir-155-RT	5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACCCCT 3’
	mir-155-Fw	5’ TTAATGCTAATTGTGATAGGGGT 3’
mir-196	mir-196-RT	5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCAAC 3’
	mir-196-Fw	5’ TAGGTAGTTTCCTGTTGTTGGG 3’
mir-296	mir-296-RT	5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAGGA 3’
	mir-296-Fw	5’ AGGGCCCCCCCTCAAT 3’
mir-21	mir-21-RT	5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAGTT 3’
	mir-21-Fw	5’ TAGCTTATCAGACTGATGTTGA 3’

2.3- Results

2.3.1- PCR Amplification of HCV NS3 and E1 Genes

E1 and NS3 cDNA were amplified from Con1/FL-Neo cells. The PCR products were confirmed by gel electrophoresis and sequencing. The PCR products of E1 (448 bp) and NS3, amplified in 2 parts, (780 and 1270 bp) are shown in Figure 2.2.

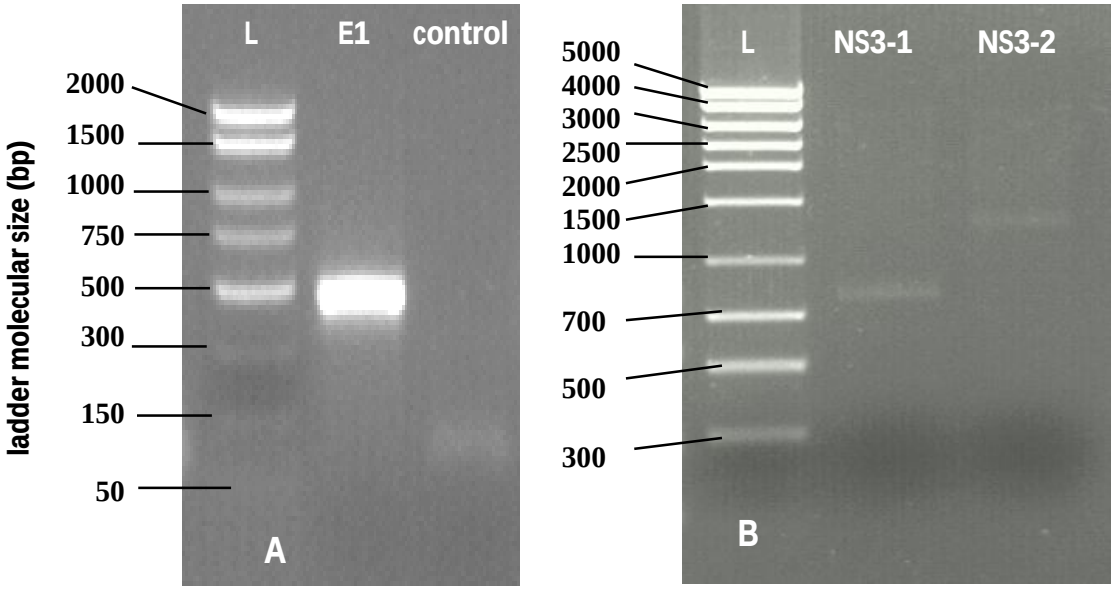


Figure 2.2: Agarose gels showing the PCR product of HCV genes. Gel (A) E1 PCR product (488 bp). L is Norgen's FastRunner ladder. Gel (B) - NS3 PCR products: NS3-1 (780 bp) and NS3-2 (1270 bp). L is Norgen's MidRanger ladder. The Control lane contains the negative control (no template). E1 and NS3 were amplified from Con1/FL-Neo cells expressing a subgenomic replicon of HCV. Size of the ladder bands are indicated in base pairs (bp).

2.3.2- Cloning of HCV-E1 and NS3 under different promoters

2.3.2.1- Construction of Ad E1A promoter plasmid

The plasmid containing Ad E1A promoter (E1AP) and E1A polyadenylation (pA) signal were designed based on pUC19. The strategy is shown in Figure 2.3. E1AP and

pA were amplified from pFG173, a plasmid containing most of the Ad5 genome. The resultant plasmid, designated as pE1P, was confirmed with sequencing.

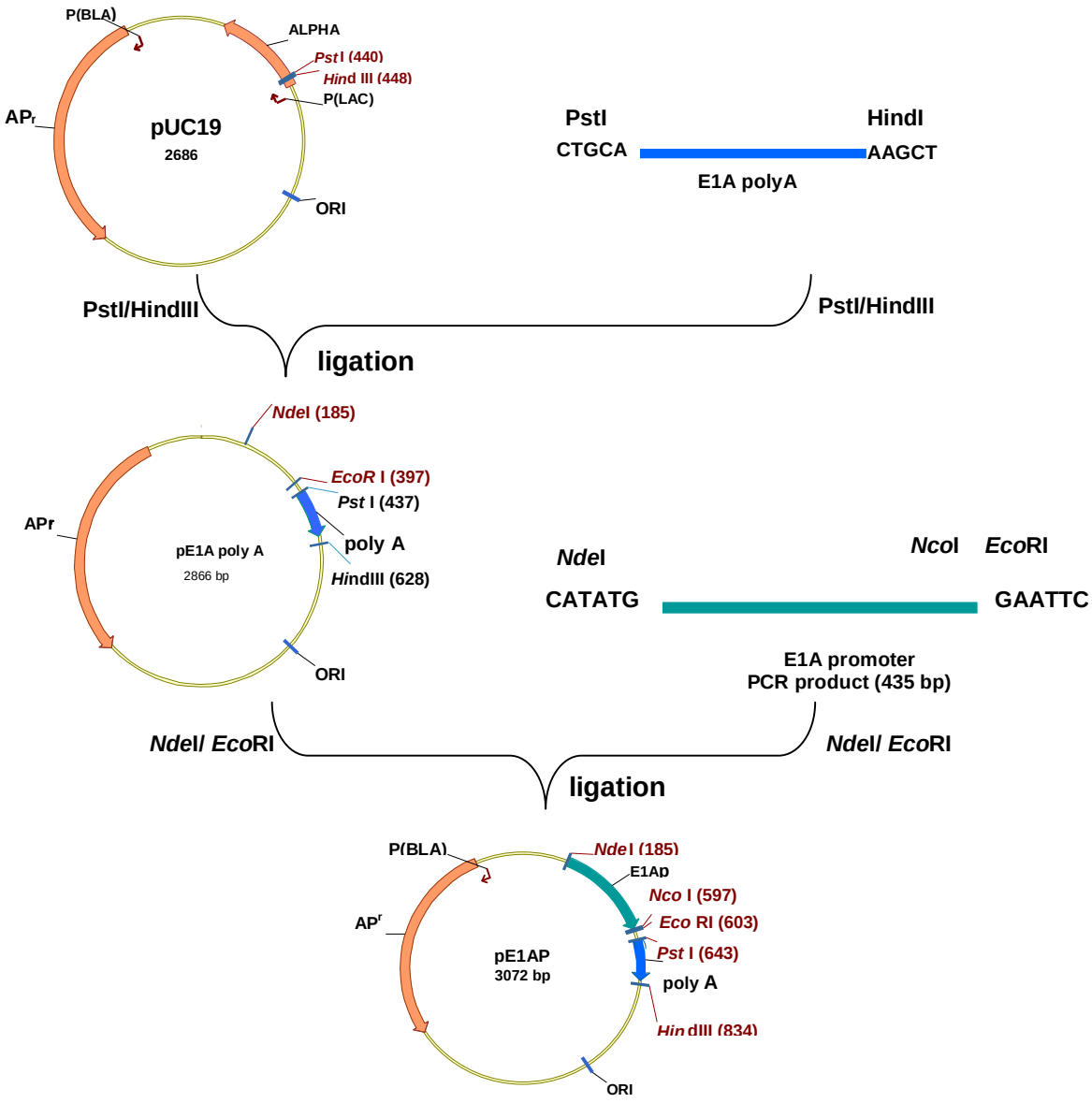


Figure 2.3: Schematic diagram showing the strategy for constructing the pE1Ap plasmid containing the adenoviral E1A promoter and polyA signal. The promoter and polyA was amplified from the adenovirus plasmid pFG173, and cloned in pUC19.

2.3.2.2- Cloning of HCV- E1 and NS3 under Ad5-E1A promoter

Two plasmids encoding HCV-E1 and HCV-NS3 under the control of Ad E1A promoter were constructed. The PCR fragments E1 or NS3 were ligated in pE1p as shown in Figures 2.4 and 2.6. The resultant plasmids, pEE1 and pENS3, were confirmed with restriction enzyme digestion and sequencing. Figures 2.5 and 2.7 show the confirmation of pEE1 and pENS3 respectively.

As shown in the gel, when plasmids pEE1 and pENS3 were digested with several restriction enzymes, the observed bands were similar to what is expected. Extra bands are possibly resultant from non-digested plasmid, e.g. a band of about under 4000 bp in lane 1 (Figure 2.5), a band of 3000 bp in lane 2 and a band of about 5000 bp in lane 3 (Figure 2.7).

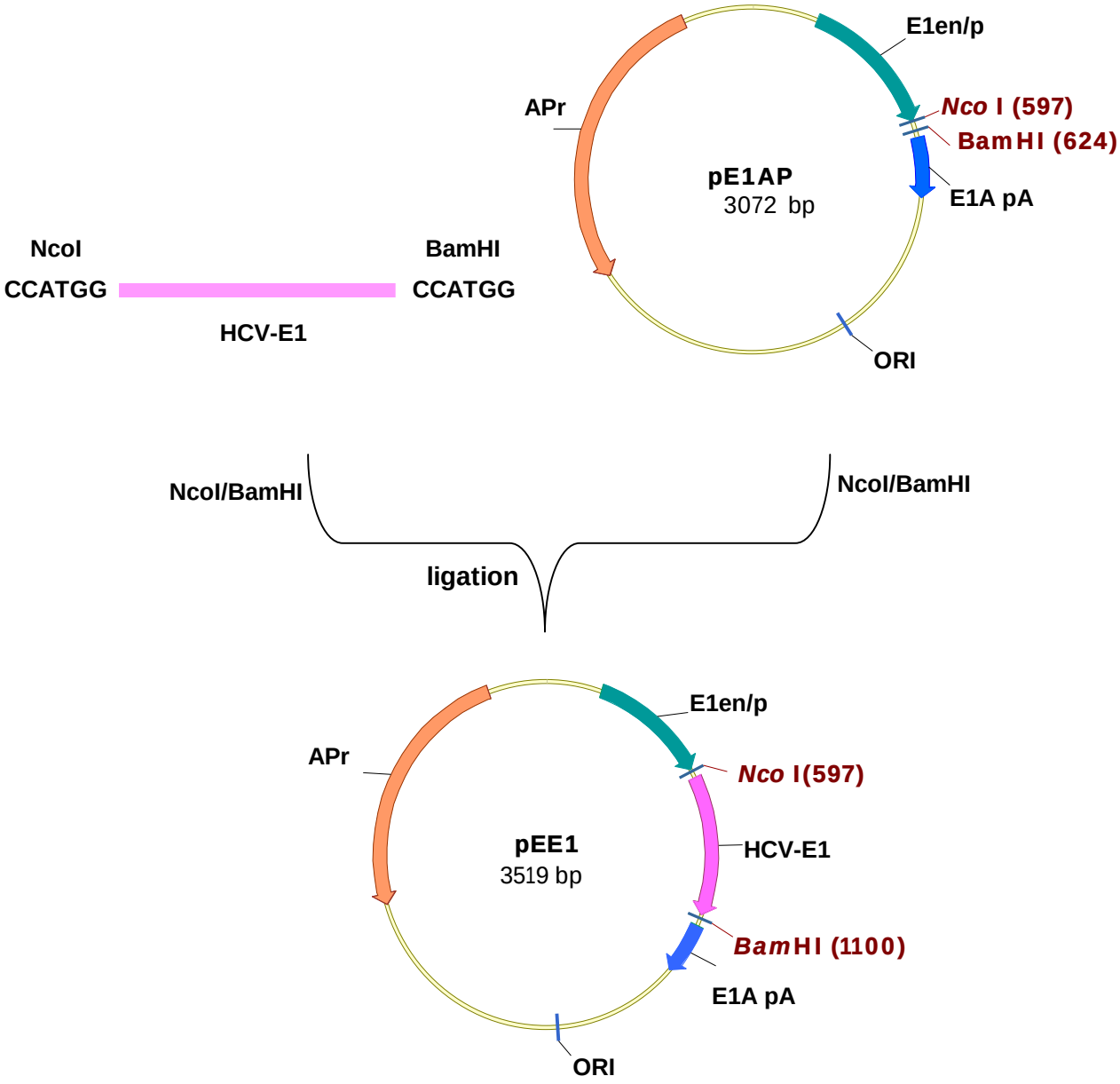
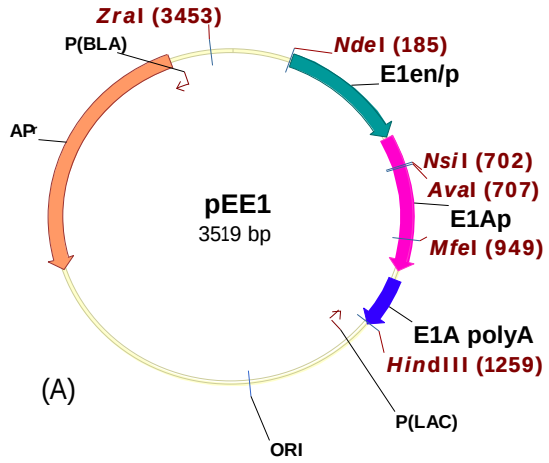


Figure 2.4: Schematic diagram showing the strategy for constructing the pEE1 plasmid. The HCV-E1 gene was amplified from Con1/Fl-Neo cells and cloned in pE1A under the control of the adenoviral E1A promoter and polyA signal.

Table 2.3: Restriction enzyme analysis of pEE1

Lane number	Enzyme	Expected band size (bp)
1	*Ava*I/ *Zra*I	2746, 773
2	*Nsi*I/ *Hin*dIII	2940, 557
3	*Nde*I/ *Mfe*I	2755, 764



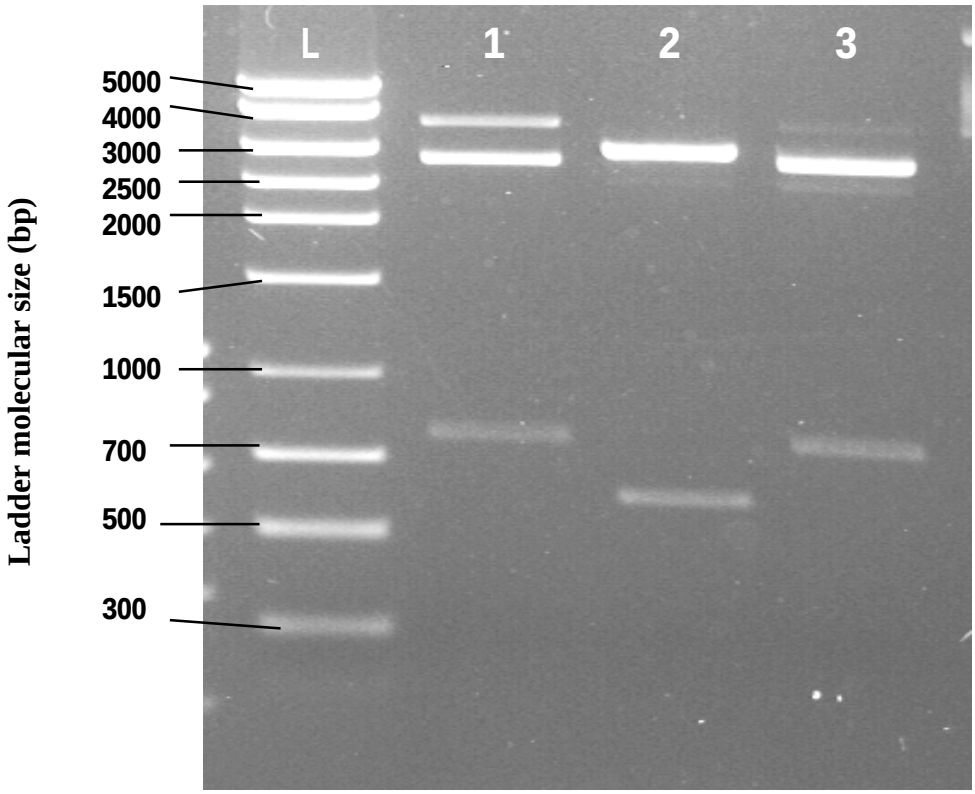


Figure 2.5: Confirmation of pEE1 by restriction enzyme digestion. (A) pEE1 plasmid map showing the restriction enzyme recognition sites. (B) Restriction enzyme digestion of pEE1on an agarose gel. L: Norgen's MidRanger ladder; Lane 1-3: plasmid DNA digested by restriction enzymes as shown in table 2.3. The size of ladder bands is shown in bp.

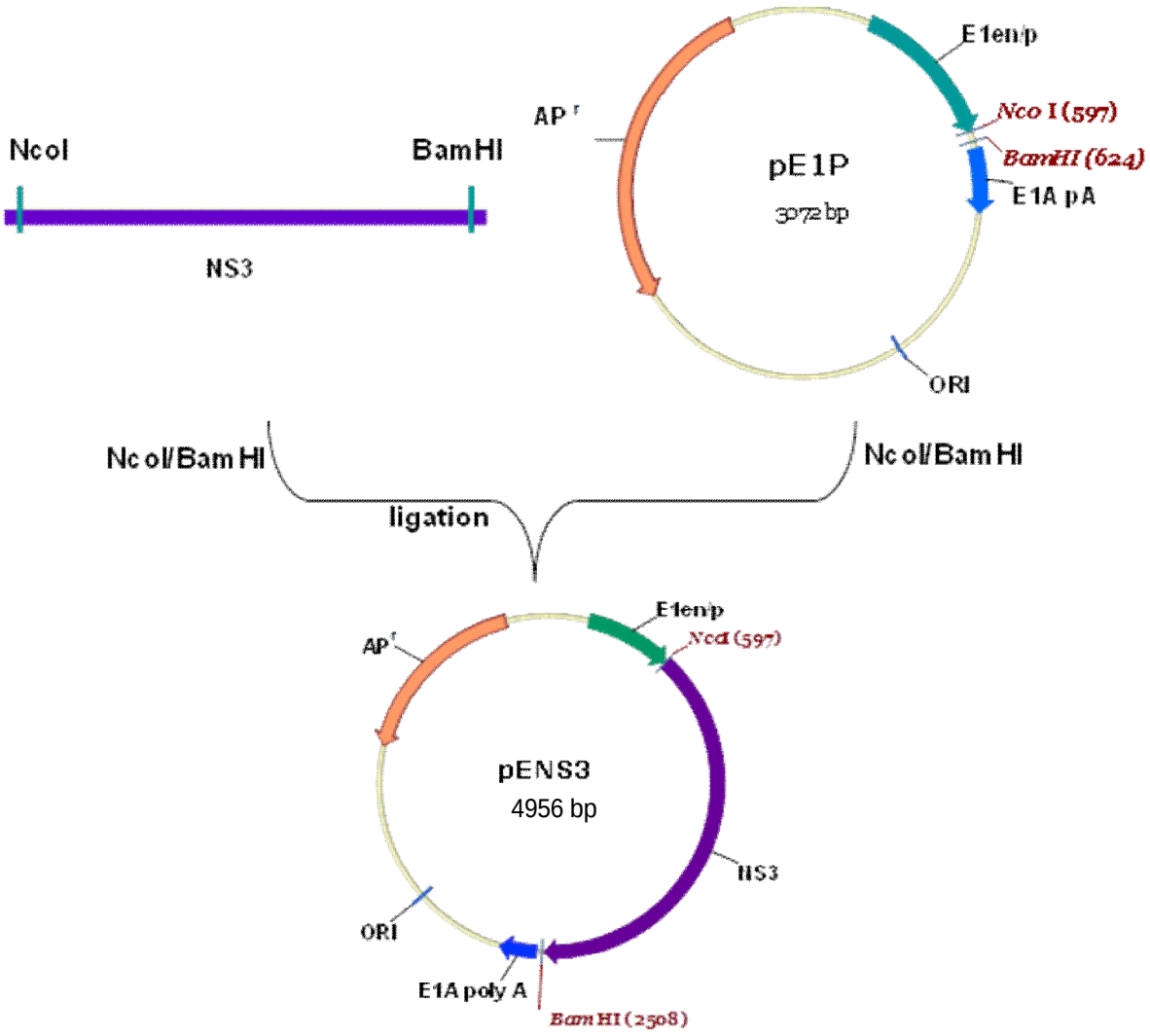
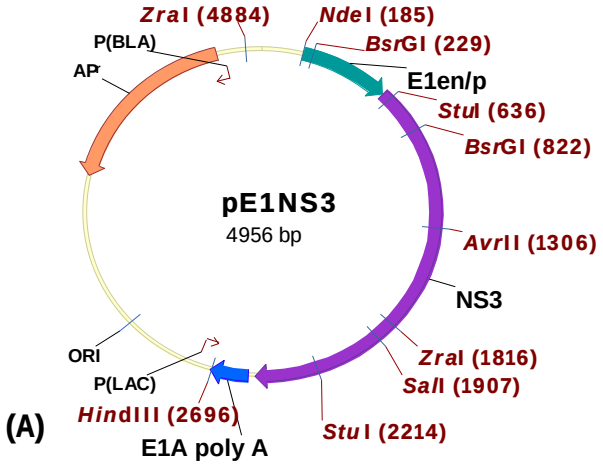


Figure 2.6: Schematic diagram showing the strategy for constructing the pENS3 plasmid. HCV-NS3 cDNA was amplified from Con1/Fl-Neo cells expressing the HCV genome, and was cloned under the control of adenoviral E1A promoter and polyA signal

Table 2.4: Restriction enzyme analysis of pENS3.

Lane	Restriction enzyme	Expected band size (bp)
1	*Hin*dIII/*Avr*II	3544/ 1390
2	*Nde*I/ *Sal*I	3234/ 1722
3	*Zra*I	3074/ 1882
4	*Bsr*G1	4363/ 593



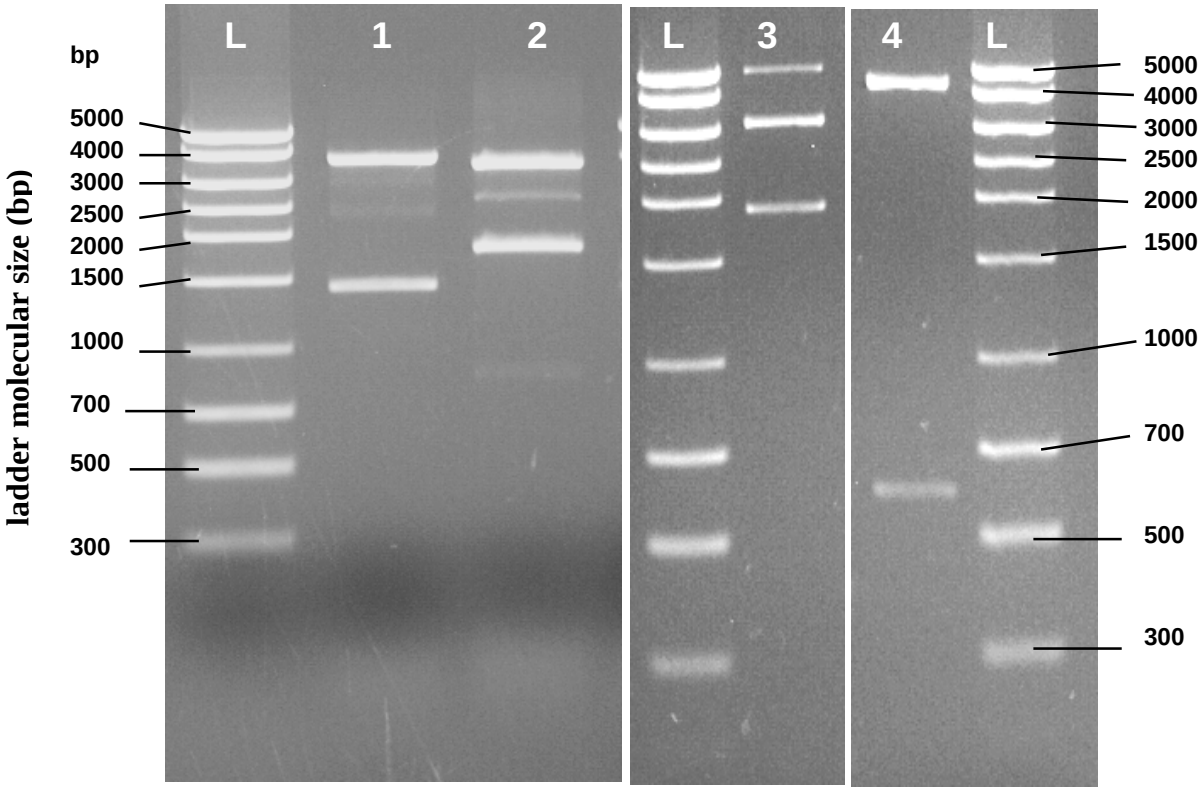


Figure 2.7: Confirmation of pENS3 by restriction enzyme digestion. (A) pENS3 plasmid map showing the restriction enzyme recognition sites. B) Restriction enzyme digestion of pENS3 on an agarose gel. L: Norgen's MidRanger marker; Lanes numbered 1-5: plasmid DNA digested by restriction enzymes as shown in Table 2.4. The size of ladder bands is shown in bp.

2.3.2.3. Cloning of HCV-E1 and NS3 under Ad5-MLP promoter

E1 and NS3 were also cloned under the control of MLP. The backbone was derived from the pMTGA plasmid, which contains Ad5-MLP followed by the Ad tripartite sequence. The polyA signal of pMTGA was derived from simian virus 40 (SV40). E1 and NS3 were cloned under control of MLP to generate pMTE1 and pMTNS3 respectively. The schematic strategies are shown in Figures 2.8 and 2.10 respectively. Both plasmids were confirmed with restriction enzyme digestion and sequencing. The digestion results of the plasmids are shown in Figures 2.9 and 2.11. The results showed that pMTE1 and pMTNS3 were digested with several restriction enzymes and the observed bands are as expected from restriction analysis. Extra bands in lanes 4 and 5 of about 4000 bp (Figure 2.9) and lane 3 of 5000 bp (Figure 2.11) possibly result from non-digested plasmid. Sequencing results showed no mutations in the constructs.

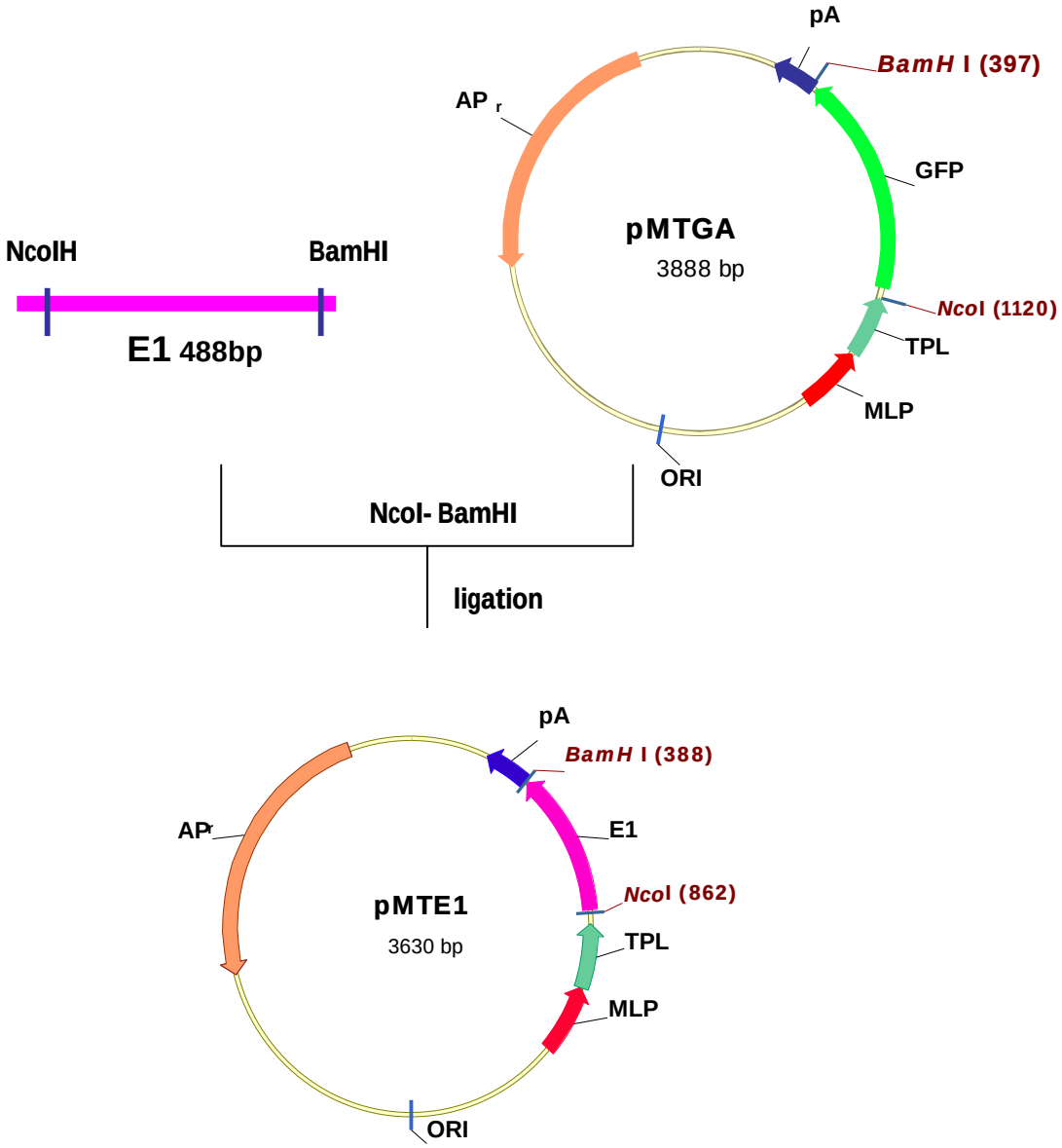
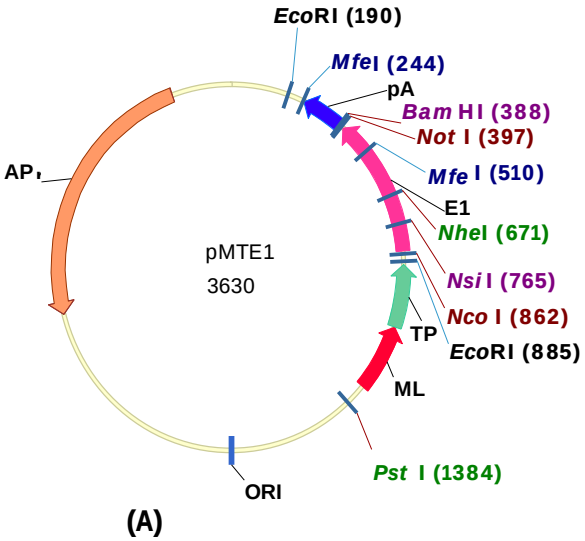


Figure 2.8: Schematic diagram showing the strategy for constructing pMTE1 plasmid. HCV-E1 gene was amplified from Con1/Fl-Neo cells and cloned under the control of the MLP promoter and SV40 polyA signal.

Table 2.5: Restriction enzyme analysis of pMTE1.

Lane	Restriction enzymes	Expected band size (bp)
1	*Eco*RI	2935, 695
2	*Mfe*I	3364, 266
3	*Nco*I, *Not*I	3165, 465
4	*Bam*HI, *Nsi*I	3253, 377
5	*Pst*I, *Nhe*I	2917, 713



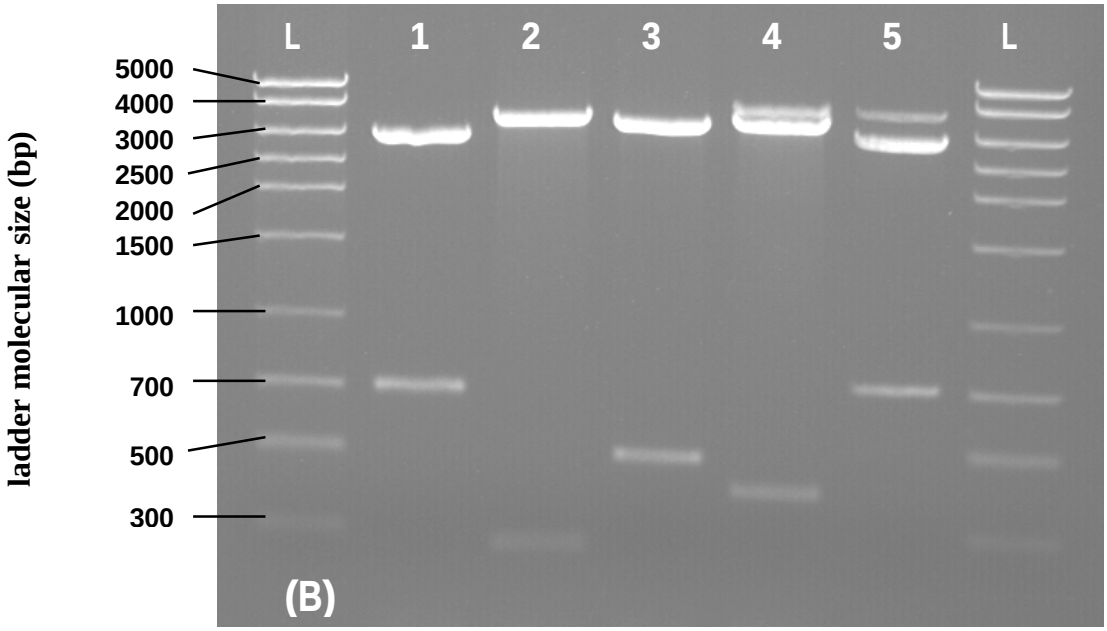


Figure 2.9: Confirmation of pMTE1 by restriction enzyme digestion. (A) pMTE1 plasmid map showing the restriction enzyme recognition sites. (B) Restriction enzyme digestion of pMTE1 on an agarose gel. L: Norgen MidRanger Ladder; Lanes numbered 1-5: plasmid DNA digested by restriction enzymes as shown in Table 2.5. The size of ladder bands is shown in bp.

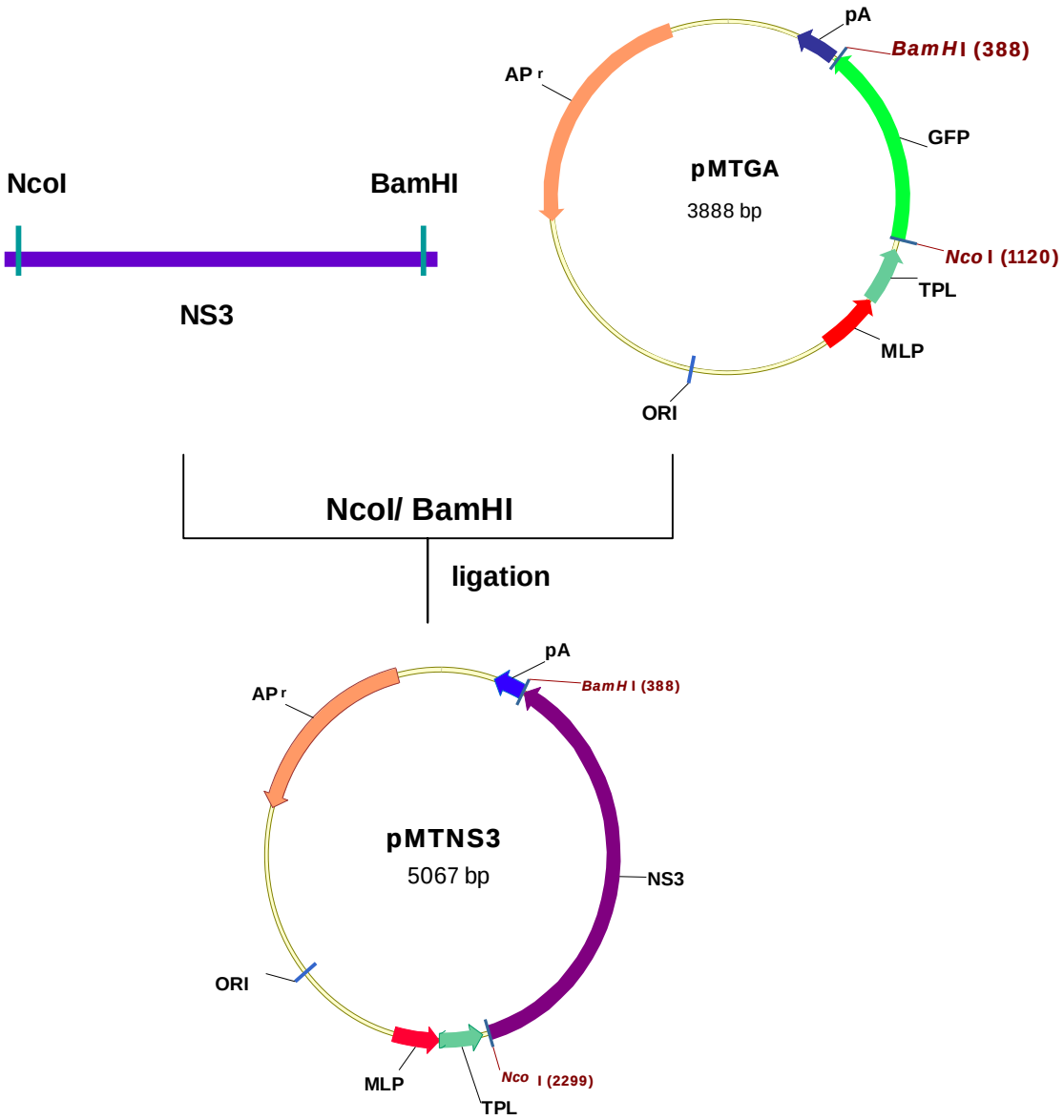
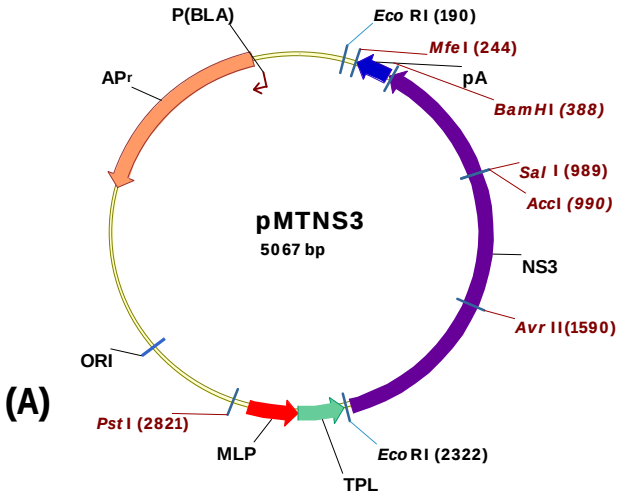


Figure 2.10: Schematic diagram showing the strategy for constructing pMTNS3 plasmid. The plasmid contains the HCV-E1 gene under the control of the MLP promoter and SV40 polyA signal.

Table 2.6: Restriction enzyme analysis of pMTNS3

Lane	Restriction enzyme	Expected band size (bp)
1	*Mfe*I, *Avr*II	3721, 1346
2	*Eco*RI, *Avr*II	2935, 1400, 732
3	*Acc*I, *Pst*I	3236, 1831
4	*Bam*HI, *Sal*I	4466, 601



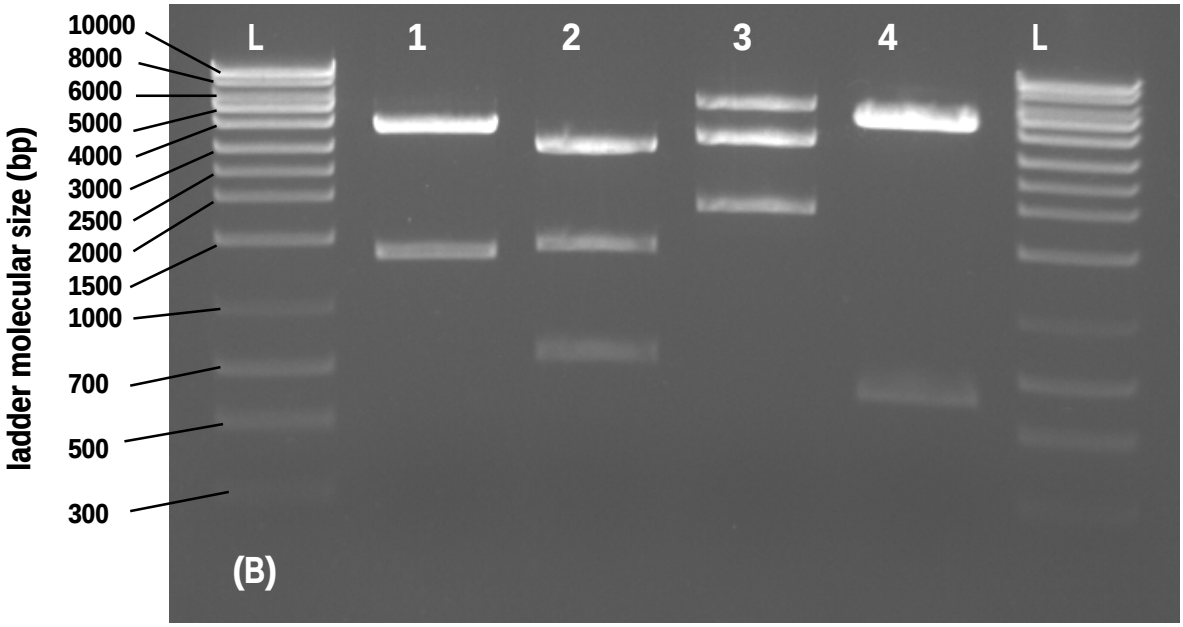


Figure 2.11: Confirmation of pMTNS3 plasmid by restriction enzyme digestion. A) The pMTNS3 plasmid map showing the position of the restriction enzyme digestion sites. B) Restriction enzyme digestion of pMTNS3 on an agarose gel. HR: Norgen's HighRanger marker, Lanes numbered 1-4: pMTNS3 plasmid digested by restriction enzymes as shown in Table 2.6. The size of ladder bands is shown in bp.

2.3.2.4- Cloning of HCV- E1 and NS3 under the CMV ie promoter

Cloning E1 and NS3under the control of the CMV ie promoter is based on the plasmid pDC-CG, which contains the CMV ie promoter, in addition to a splicing donor/ acceptor signal and polyA signal from SV40. The newly formed plasmids were designated as pDCE1and pDCNS3. The plasmids were confirmed by restriction enzyme digestion and sequencing. The strategy for construction of pDCNS3 and pDCE1 is shown in Figure 2.12 and the restriction maps for both plasmids were shown in Figures 2.13 and 2.14 respectively. The results show that pDCE1 and pDCNS3 were digested with several restriction enzymes and the observed bands are similar to what is expected from restriction analysis. Sequencing results showed no mutations in the constructs.

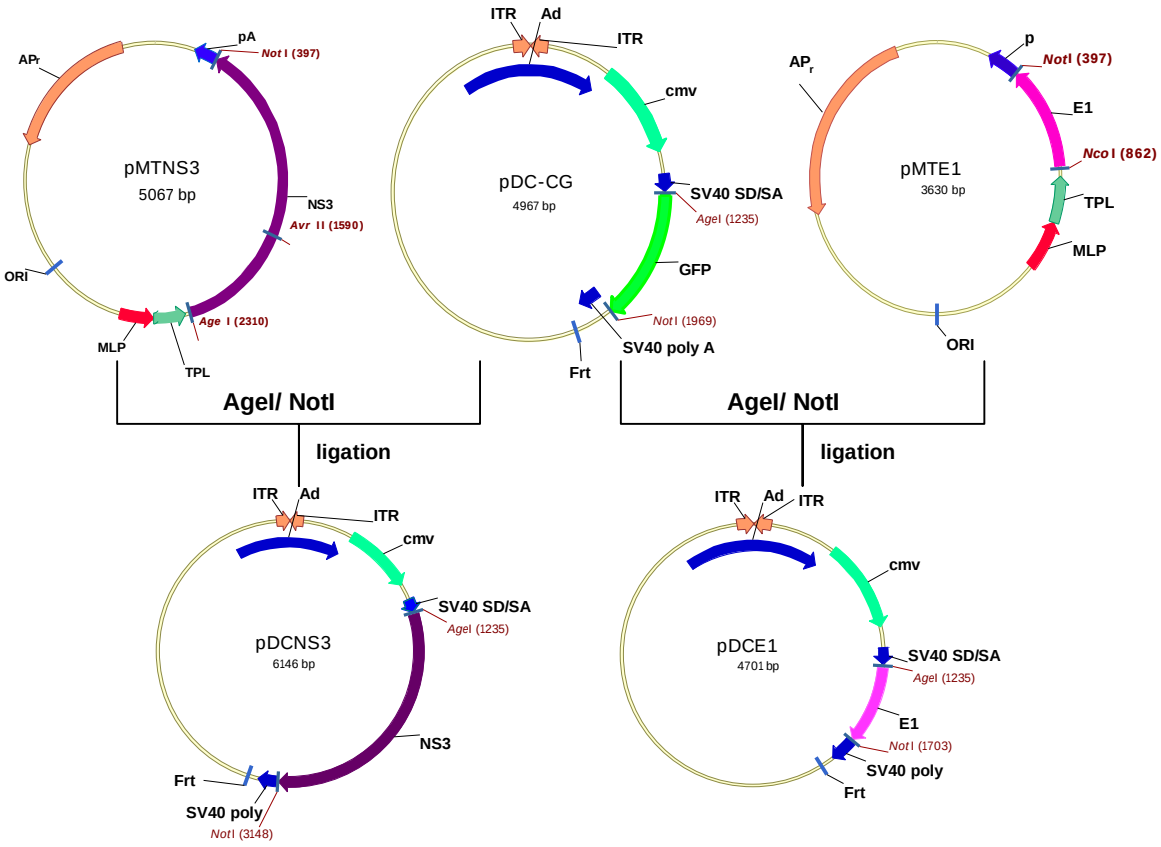


Figure 2.12: Schematic diagram showing the strategy for the construction of the pDCNS3 and PDCE1 plasmids. NS3 and E1 genes were digested from pMTNS3 and pMTE1 respectively, with *AgeI* and *NotI*. They were then cloned under the control of the CMV ie promoter and SV40 polyA signal in the pDC-CG plasmid after digestion with *AgeI* and *NotI*, releasing GFP.

Table 2.7: Restriction enzyme analysis of pDCNS3.

Lane	Restriction enzymes	Expected band size (bp)
1	*Sal*I	5335/811
2	*Stu*I	4568/1578
3	*Kpn*I/*Avr*II	5427/719
4	*Age*I/*Not*I	4233/1913

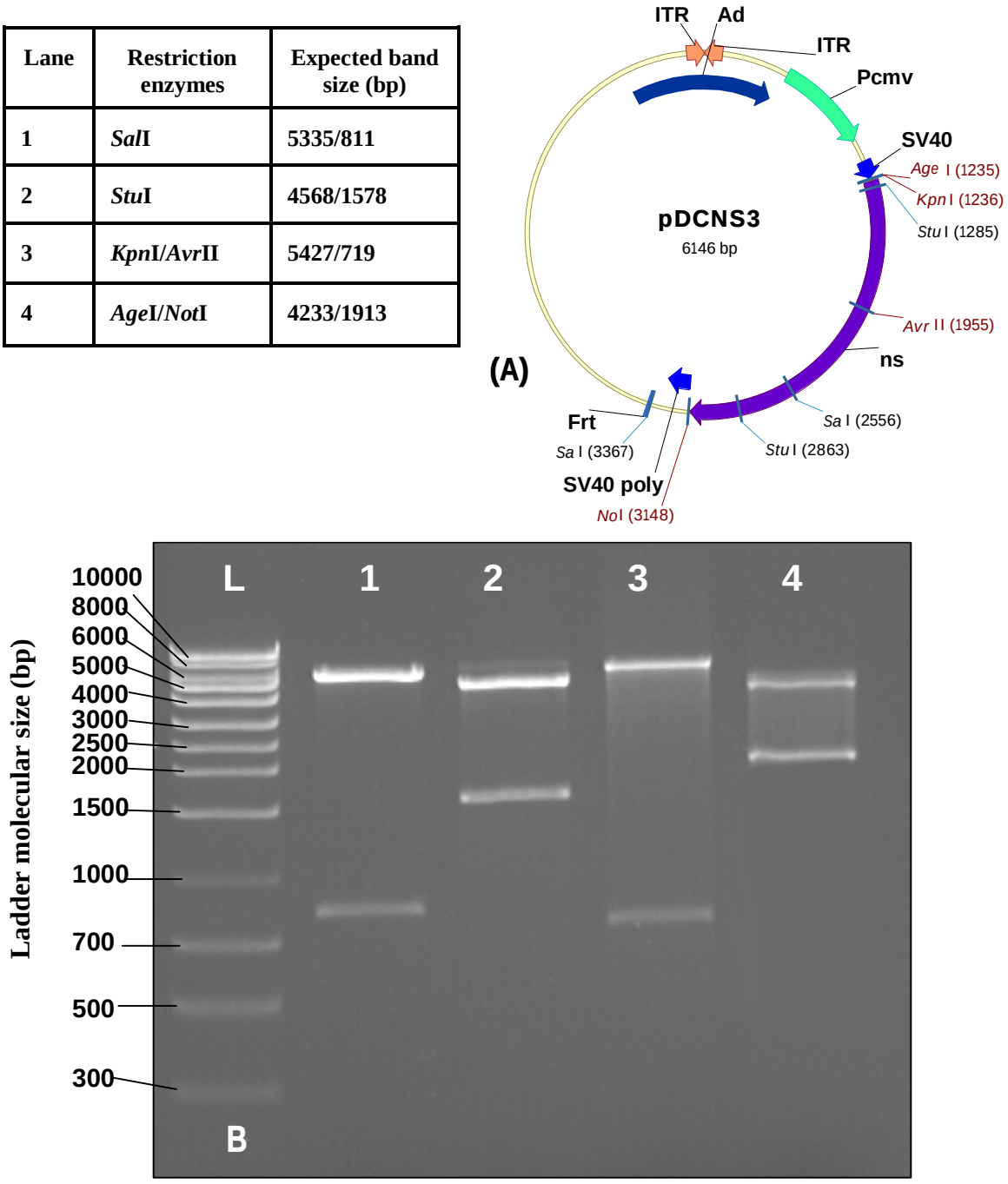


Figure 2.13: Confirmation of pDCNS3 with restriction enzymes. A) pDCNS3 plasmid map showing the position of restriction enzyme recognition sites. B) Restriction enzyme digestion of pDCNS3 on an agarose gel. L: Norgen's HighRanger ladder; Lanes numbered 1-4: plasmid DNA digested by restriction enzymes as shown in Table 2.7. The size of ladder bands is shown in bp.

Table 2.8: Restriction enzyme analysis of pDCE1

Lane	Restriction enzymes	Expected band size (bp)
1	*Ava*I	4795 / 242 bp
2	*Mfe*I	4771 / 266 bp
3	*Kpn*I/*Nhe*I	4849 / 188 bp
4	*Eco*RI/*Nsi*I	4161 / 876 bp

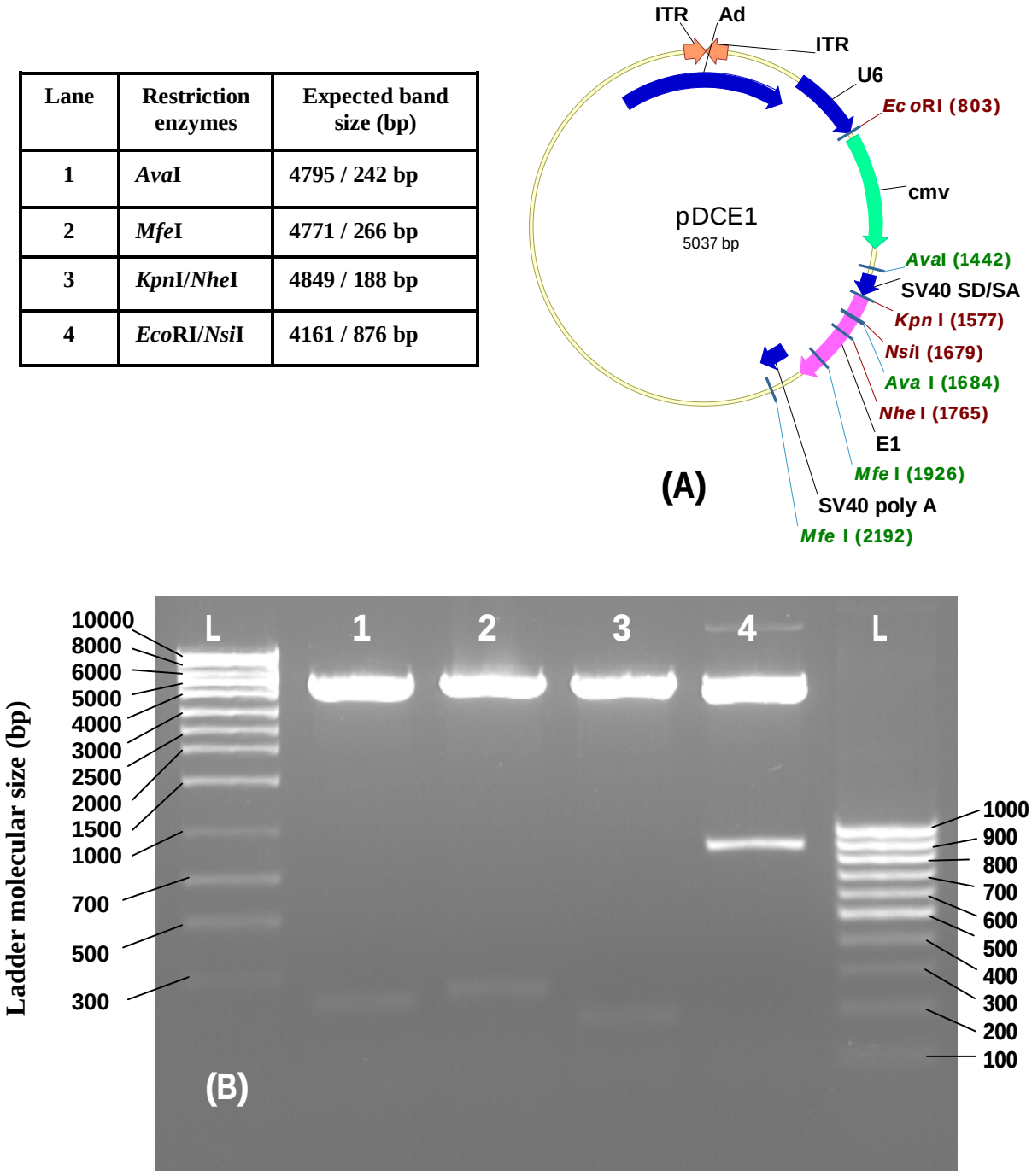


Figure 2.14: Confirmation of pDCE1 with restriction enzymes. (A) pDCE1 plasmid map showing the position of the restriction enzyme recognition sites. B) Agarose gel demonstrating the restriction enzyme digestion of pDCE1. First and last Lanes: Norgen HighRanger and PCRsizer markers respectively. Lanes 1-4: plasmid DNA digested by restriction enzymes as shown in Table 2.8. The size of ladder bands is shown in bp.

2.3.3- *In-vitro* evaluation of the expression of HCV-NS3 and E1

2.3.3.1- Confirmation of the vaccines' expression activity

The expression of NS3 and E1 genes was confirmed in HEK 293 cells transfected with the constructed plasmids. The expression was determined using RT-PCR which indicates successful expression of both genes, NS3 (283 bp) (Figure 2.15) and E1 (448 bp) (Figure 2.16) under the control of the three promoters CMV ie, MLP and E1A. These results confirmed the activity of the transcription of NS3 and E1 genes.

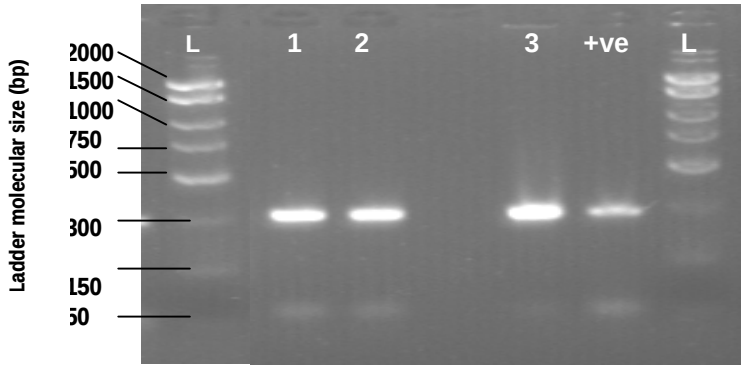


Figure 2.15: Expression of NS3 24 h post-transfection in HEK 293 cells with different promoter plasmids, MLP (lane1), E1A (lane 2) and CMV ie (lane 3). PCR products of NS3 are shown (283 bp). L: Norgen's FastRunner. Size of each ladder band is shown in bp. +ve is a positive control amplified from plasmid encoding NS3.

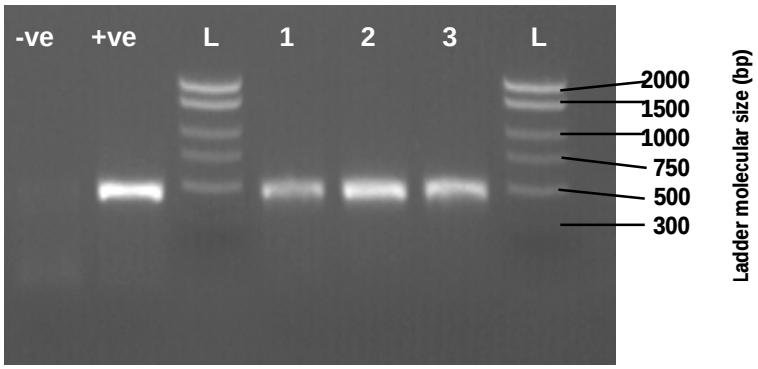


Figure 2.16: Expression of E1 24 h post-transfection in HEK 293 cells with different promoter plasmids, MLP (lane 1), CMV ie (lane 2) and E1A (lane 3). PCR products of E1 are shown (448 bp). L: Norgen's FastRunner. Size of each ladder band is shown in bp. –ve is a negative control with no template, +ve is a positive control amplified from plasmid encoding E1.

2.3.3.2- Comparison of the transcription levels of HCV E1 and NS3 plasmids

2.3.3.2.1- The transcription level of E1 driven by the different promoters

The activity of three different promoters, CMV ie, E1A and MLP were compared in HEK 293 cells at different time points post-transfection, including 0, 12, 24 and 48 h. The expression levels for E1 were quantified using qRT-PCR. The E1 mRNA copy number was obtained using a standard curve of known plasmid concentration. The results show that the CMV ie promoter has the highest activity starting at 12 h, which increased at 24 h and reached a maximum at 48 h. At 12 h, both MLP and E1A demonstrated very low transcription levels, which increased to maximum activity at 24 h, then decreased slightly at 48 h. The mRNA copy number/ cell for CMV ie was significantly higher than for MLP and E1A at all points. The mRNA copy number/cell for E1A was higher than for MLP, but the difference was not significant (Figure 2.17).

2.3.3.2.2- The transcription level of NS3 driven by the different promoters

The transcription levels of NS3 were determined by qRT-PCR at different time points post-transfection of HEK 293 cells, including 0, 12, 24 and 48 h. The same trend was seen as that of E1. CMV ie demonstrated the highest transcription activity followed by E1A and then MLP. The mRNA level driven by CMV ie was higher than MLP and E1A promoters at all time points. Transcription level was low at 12 h and increased gradually over time at 24 h and 48 h. mRNA levels from both E1A and MLP reached a peak at 24 h and decreased at 48 h (Figure 2.18).

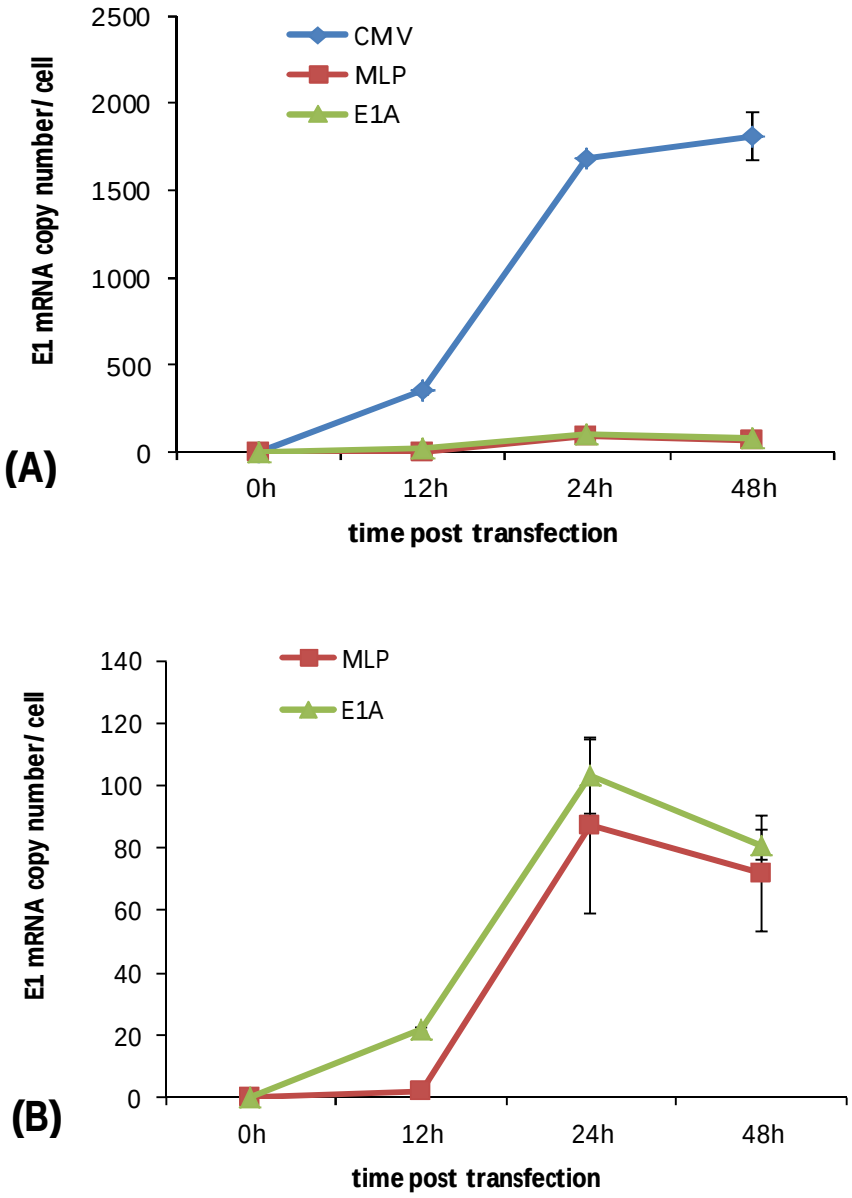


Figure 2.17: The level of E1 transcription under different promoters (E1A, MLP, CMV ie), over 0, 12, 24, 48 h post-transfection in 293 cells. Copy numbers were obtained by qPCR using a standard curve of known plasmid DNA concentration. Graph (A) Comparison of the 3 promoters. Graph (B) Comparison of the 2 promoters E1A and MLP enlarged. Error bars represent the standard deviation. N=3.

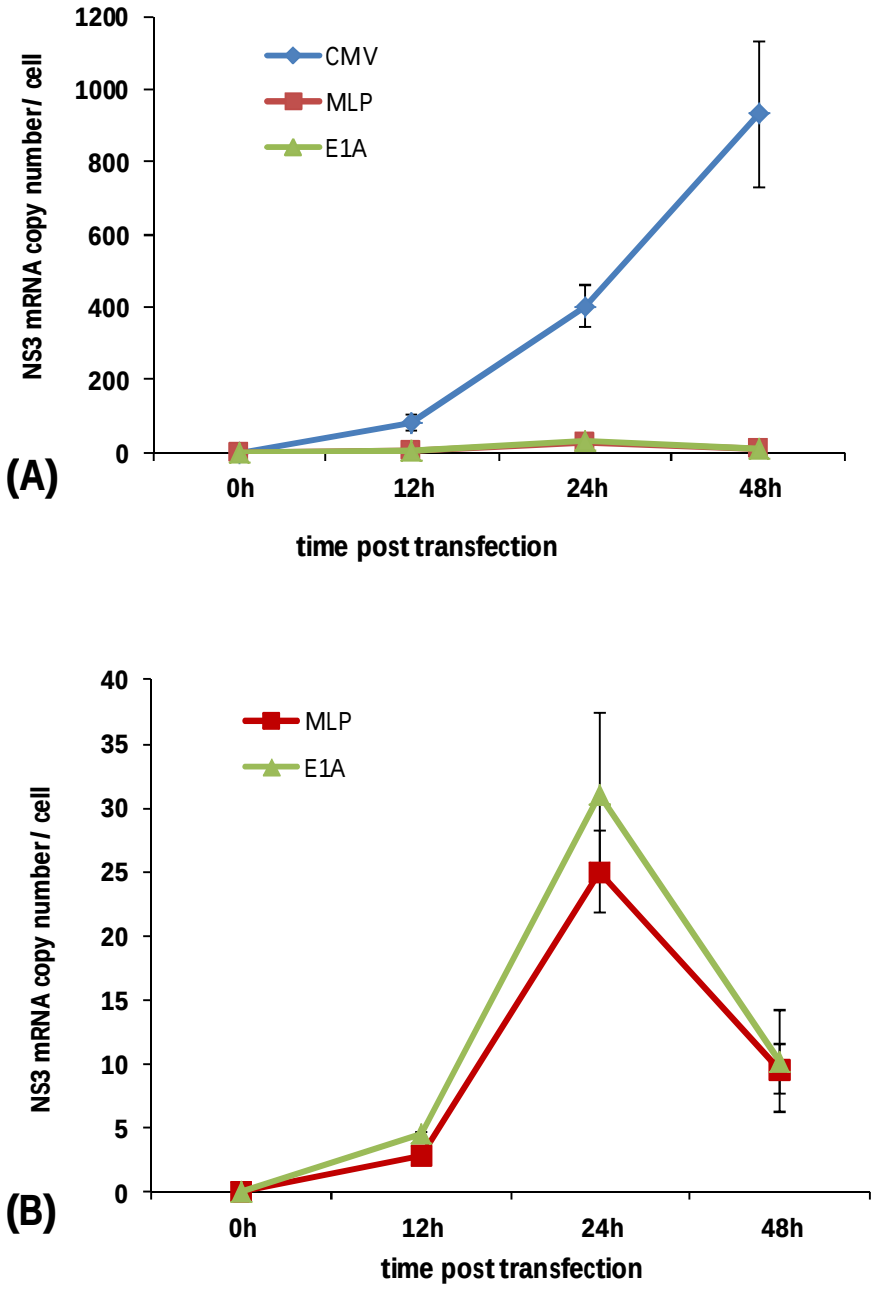


Figure 2.18: The level of NS3 transcription under different promoters (E1A, MLp, CMV ie), over 0, 12, 24, 48 h post-transfection in 293 cells. Copy numbers were obtained by qPCR using a standard curve of known plasmid DNA concentration. Graph (A) Comparison of the 3 promoters. Graph (B) Comparison of the 2 promoters E1A and MLP enlarged. Error bars represent the standard deviation. N=3.

2.3.4- *In vitro* expression of E1 and NS3 proteins in *E. coli*

2.3.4.1- Construction of bacterial expression plasmids pT7E1 and pT7NS3

Recombinant E1 and NS3 proteins were expressed in *E. coli* in order to be used for the detection of E1 and NS3 antibodies induced by vaccination. First, NS3 and E1 were cloned under the T7 promoter. The resultant plasmids, pT7E1 and pT7NS3 (Figure 2.19), were screened by *AfeI* enzyme digestion. The digestion showed the correct bands as expected (Figures 2.20, 2.21). The plasmids were then confirmed by sequencing.

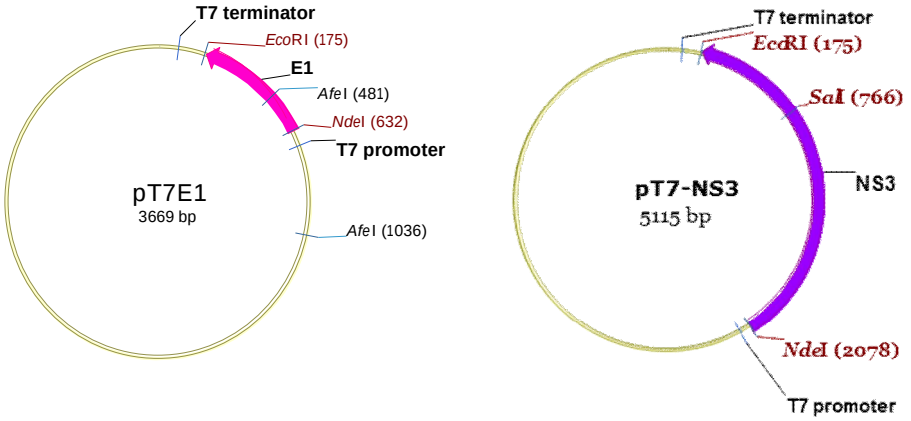


Figure 2.19: Schematic diagram showing the expression plasmids pT7E1 and pT7NS3

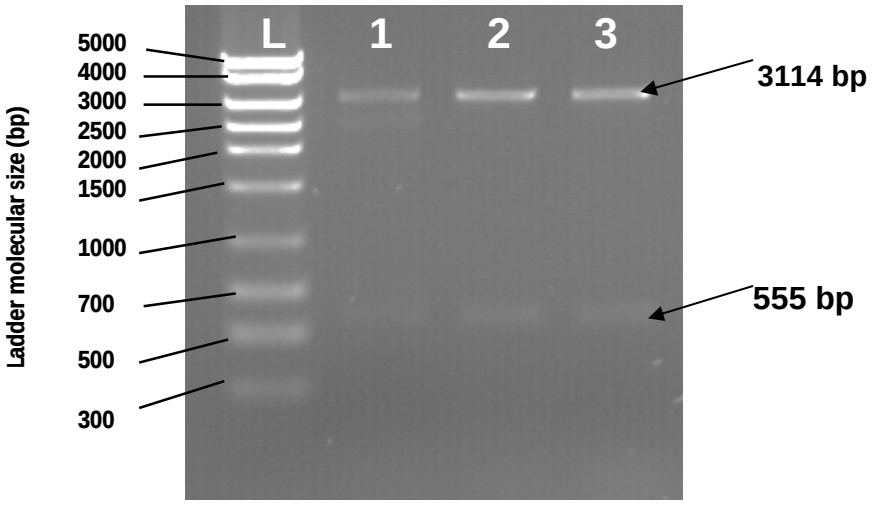


Figure 2.20: Agarose gel showing the confirmation of pT7E1 plasmids with *AfeI* digestion. L: Norgen's MidRanger. Size of ladder bands is shown in bp. The expected bands 3114 and 555 bp were obtained.

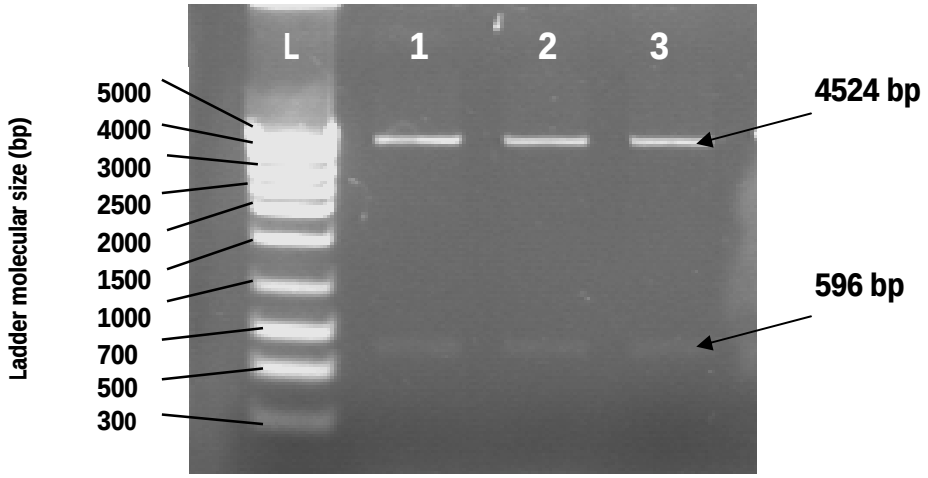


Figure 2.21: Agarose gel showing pT7NS3 plasmids confirmed with *EcoR1* and *SalI* digestion. L: Norgen's MidRanger. Sizes of the ladder bands are shown in bp. The expected bands 4524 and 596 bp were obtained.

2.3.4.2- Expression and isolation of E1 and NS3 proteins

The expression plasmids pT7E1 and pTNS3 were transformed into *E. coli plysS*. E1 and NS3 proteins were isolated from the inclusion bodies. The isolated proteins were separated by SDS-PAGE. The gel showed the expression of E1 protein (16.5 kDa; Figure 2.22) and NS3 protein (68 kDa; Figure 2.23), with the correct bands but with minor bacterial protein contamination. The bacterial lysate from non-transformed *E. coli plysS* was loaded as a negative control. The control did not show the NS3 and E1 bands.

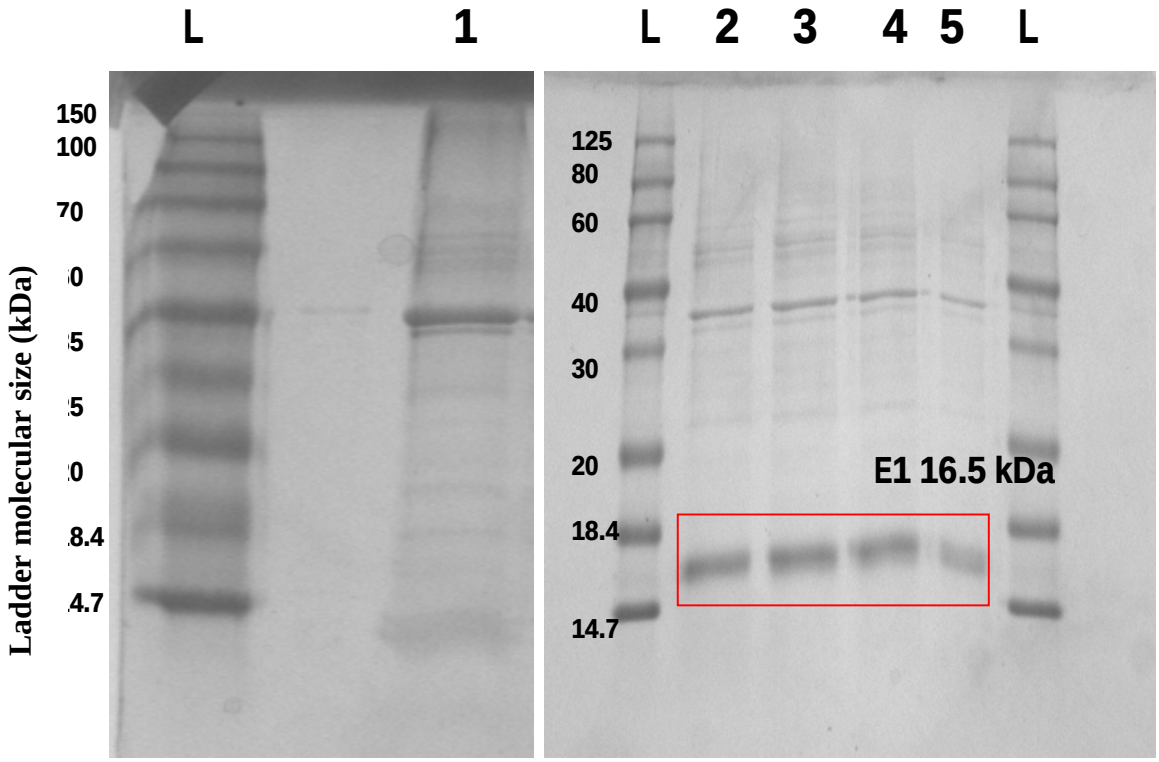


Figure 2.22: SDS-PAGE gel showing E1 (16.5 kDa) expressed and isolated from *E.coli* (Lanes 2-5) compared to the non-transformed *E.coli plysS* (Lane 1) as a control (band of 16.5 kDa is absent). L: Norgen's Proteoladders. The sizes of the ladder bands are shown in kDa**.**

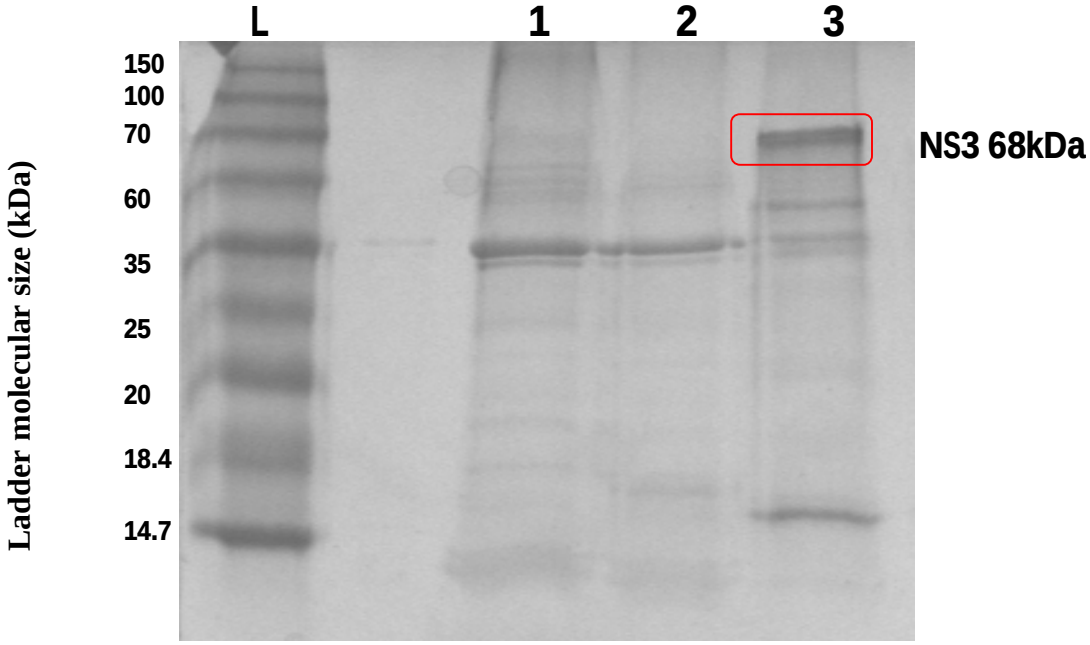


Figure 2.23: SDS-PAGE showing NS3 (68 kDa) expressed and isolated from *E. coli* (Lane 3) compared to the non-transformed *E. coli plysS* (Lanes 1, 2) as a control (band of 68 kDa is absent). L: Norgen Proteoladder 150. The sizes of the ladder bands are shown in kDa.

2.3.5- The effect of different promoters on the efficacy of the vaccine

2.3.5.1- Evaluation of HCV-E1 and NS3-specific antibody levels

To investigate the effect of different promoters on the immune response, sera from immunized and control mice were collected 2 weeks after the last dose. The antibody level was measured using ELISA. All immunized animals produced antibodies against the HCV E1 and NS3 proteins. The specificity of the antibodies was confirmed by western blot. The results demonstrated high titers of HCV E1 and NS3-specific antibodies in animals injected with plasmids controlled by the CMV ie promoter. On the other hand, plasmids that contain Ad MLP and Ad E1AP resulted in lower antibody levels but were still higher than the control animals (Figures 2.24 and 2.25). The western blot confirmed the presence of E1 and NS3 antibodies shown by signal at the right band sizes. There were also extra cross reactive bands (Figures 2.26 and 2.27).

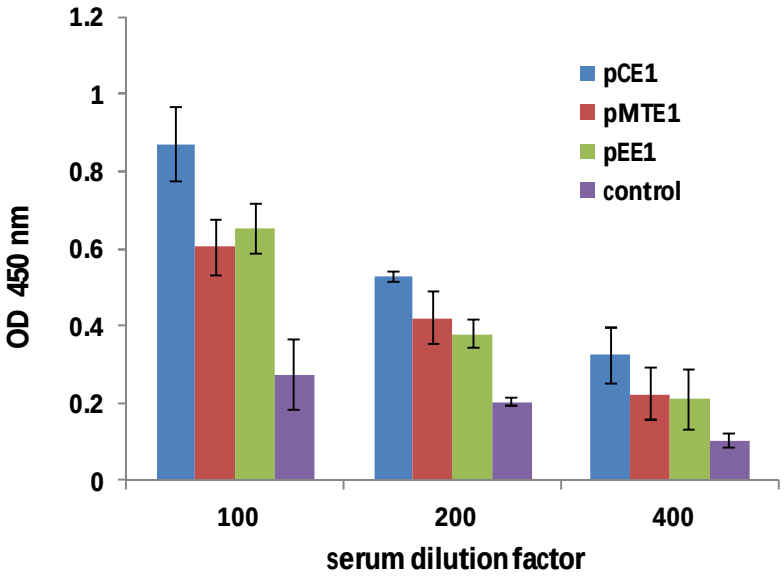


Figure 2.24: E1 antibody levels in the sera of animals immunized with E1 plasmids under different promoters: CMV ie (pCE1), MLP (pMTE1) and E1A (pEE1). The antibody levels were measured, 2 weeks after immunization, by OD at 450 nm in different serum dilutions using ELISA. Control animals were injected with PBS. Bars represent standard deviation, n = 3.

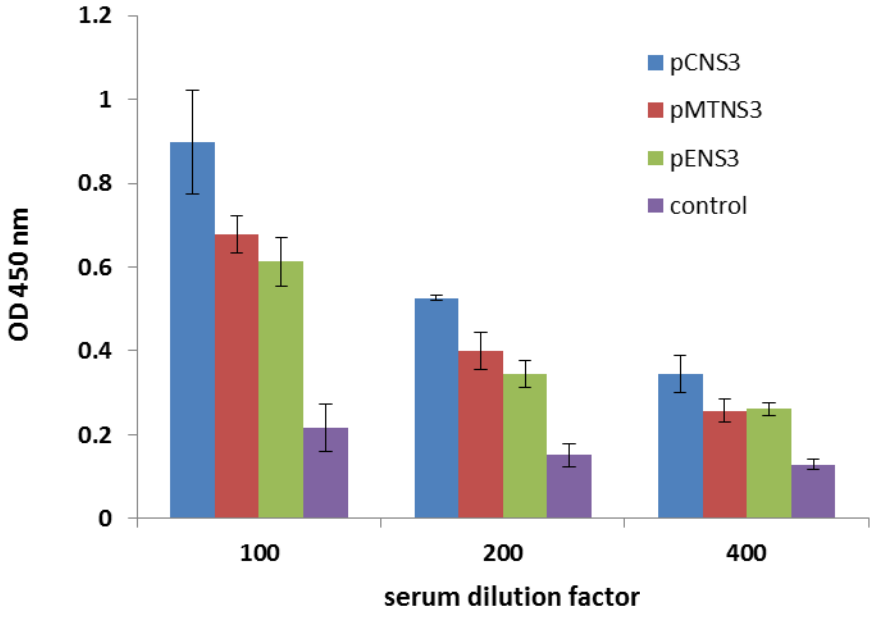


Figure 2.25: NS3 antibody levels in sera from animals immunized with NS3 plasmids under different promoters: CMV ie (pCNS3), MLP (pMNS3) and E1A (pENS3). The antibody levels were measured, 2 weeks after immunization, by OD at 450 nm in different serum dilutions using ELISA. Control animals were injected with PBS. Bars represent standard deviation, n = 3.

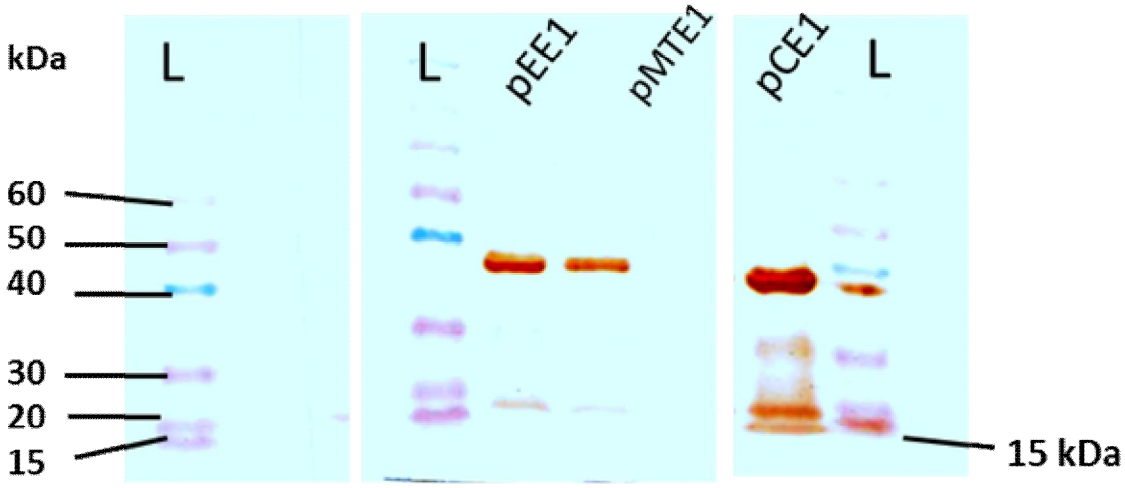


Figure 2.26: Western blot of mice groups immunized with E1 plasmids under different promoters: CMV ie (pE1), MLP (pME1) and E1A (pEE1) compared to non immunized mice. A band around 16 kDa showed the presence of E1 antibodies.

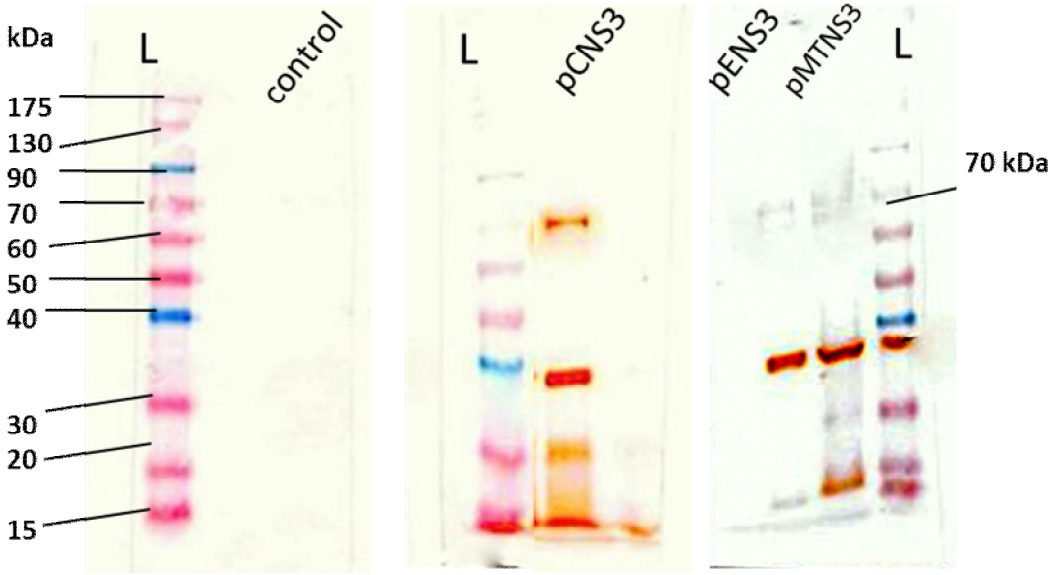


Figure 2.27: Western blot of mice groups immunized with NS3 plasmids under different promoters: CMV ie (pCNS3), MLP (pMTNS3) and E1A (pENS3) compared to non immunized mice. A band around 70 kDa showed the presence of NS3 antibodies**.**

2.3.5.2- The effect of vaccination on serum miRNA levels

Cellular miRNAs are essential in the regulation of all cellular activities and they determine the fate of several cell types. The purpose of this experiment was to investigate the relationship between the immune response and cellular miRNA. The levels of mir-155, mir-181, mir-21, mir-196 and mir-296 were examined in animals immunized with E1 and NS3 plasmid vaccines. The results demonstrated an upregulation of certain miRNAs. mir-181 expression was upregulated in all immunized groups except the pCNS3 injected group, however the highest expression was observed in the groups immunized with pEE1. mir-21 was also upregulated in groups immunized with pMTNS3, pMTE1 and pEE1. Mir-296 showed a slightly elevated expression in those vaccinated groups that received pMTNS3 and pMTE1. However the increase in miRNAs was not statistically significant ($p > 0.05$). The results are shown in Figure 2.28. mir-155 and mir-196 did not result in detectable Ct values (results not shown).

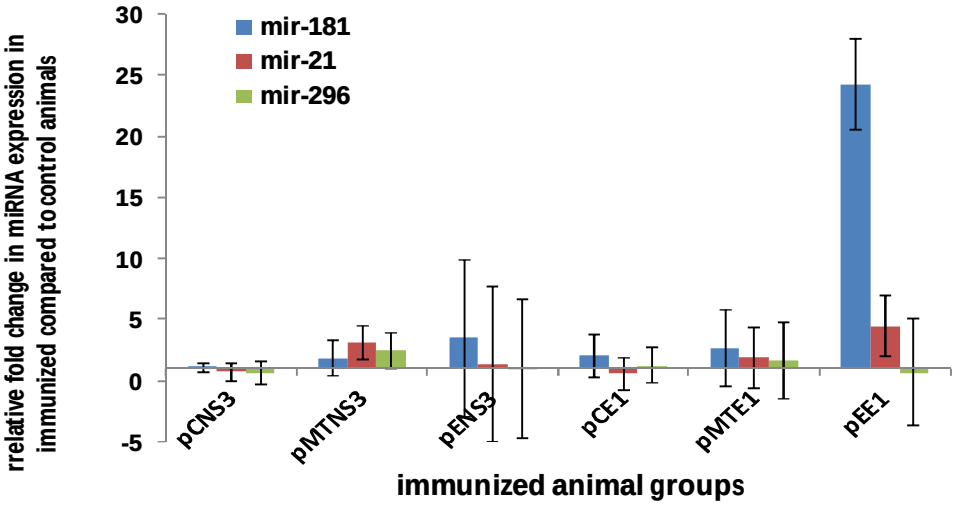


Figure 2.28: The expression level of miRNAs 2 wks after mice immunization with E1 and NS3 plasmids. The levels of mir-181, mir-21 and mir-296 in the sera of immunized animals are expressed as a relative change in fold compared to control animals (injected with saline) measured by ΔΔCt method. Error bars represents standard deviations, (n=3). Results are considered significant if p value < 0.05.

2.4- Discussion

DNA vaccination is a promising approach for challenging viruses. In addition to its ability to induce both cellular and humoral immunity, DNA vaccine technology allows plasmid modifications to enhance or broaden the immune response.

However, DNA vaccine trials have shown limited immunogenicity in humans compared to small experimental animals. Therefore, optimization of the DNA vaccines is critical to enhance their efficacy. The focus of this thesis is to optimize antigen expression by using different promoters. Two promoters that were derived from adenovirus, the weak early promoter E1A and the strong late promoter MLP, were evaluated. These promoters were chosen in order to compare between early and late promoters and because they were not previously evaluated in DNA vaccine although they were evaluated for *in vitro* expression.

2.4.1- *In-vitro* expression of HCV E1 and NS3 under different promoters

Antigen expression under different promoters was evaluated *in vitro.* The results demonstrated a higher transcription level of NS3 and E1, driven by the CMV ie promoter compared to MLP and E1A promoters. This can be explained by the known strength of the CMV ie promoter, due to the strong TATAA box, enhancers located upstream of the transcription initiation site, as well as its cyclic-AMP response elements (Boshart *et al.*, 1985; Hunninghake *et al.*, 1989). This result is consistent with other studies that compared different promoters and found that CMV ie was more effective in transgene expression (Zheng and Baum, 2005; Papagatsias *et al.*, 2008). On the other

hand, both Ad promoters exhibited significantly lower transcriptional activity (mRNA level), which is in accordance with the results shown by Goossens *et al.,* (2000), where they found that the CMV ie promoter induced 6-10 fold higher activity in reporter gene expression than MLP. Another experiment using adenovirus to express IL2 under MLP showed less expression than both RSV and CMV ie (Griscelli *et al.,* 2003). In the case of the E1A promoter, it has been shown that its activity is 100 fold lower than the CMV ie promoter in neuronal cells (Smith *et al.,* 2000). Moreover, Ad vectors induced expression of the E1A proteins under the CMV ie promoter 10 fold higher than its endogenous E1A promoter (Zheng *et al.,* 2005).

The results showed that there was no significant difference between the activity of E1A and MLP, which coincides with the fact that the strong activity of MLP is related to late infection, resulting from the interaction of virus-induced transcription factors with the binding sites downstream to the promoter. This is confirmed by deletion of these sites which reduced transcription activity by 25-50 fold (Leong *et al.,* 1990). It has been reported that Ad E1A protein supplied by HEK 293 cells can transactivate some promoters such as MLP and CMV ie (Cockett *et al*., 1990) and reduce the activity of the E1A promoter (Schaack *et al*., 2001). However our results showed that both E1A and MLP promoters have similar activity in HEK 293 cells, which agrees with the finding in natural infections that MLP demonstrates similar activity to the early promoters in the early phase (Nevins, 1981; Shaw and Ziff, 1980). This indicates that HEK 293 cells can be used for monitoring Ad promoters' activity, and the expression of E1 has minimal effects on their activity.

In general, these results indicate that the CMV ie promoter is more effective in driving antigen expression. Although MLP is a strong promoter during viral infection, it is less effective when it is used alone. E1A is a weak promoter, and therefore induced less activity. All promoters reached the maximum activity at 24 h as they need time for the accumulation of the mRNA to reach a detectable level. We did not monitor more points since we wanted to compare the activities of different promoters rather than the duration of their activity, however the mRNA level is expected to reduce gradually due to cell division and plasmid dilution. Even in animal immunization with plasmid vaccine, the antigen expression duration is not long enough so booster doses are usually needed.

These lines of experiments demonstrated that weak but early promoters did not have an advantage over a strong promoter regardless if it is immediate, early or not. These results confirmed that the strength of the promoter is more important than temporal expression.

2.4.2- Evaluation of the immune response against the vaccine constructs

In order to investigate the correlation between the level of protein expression and humoral immune responses elicited by the HCV vaccine constructs, the development of E1 and NS3 antibodies in immunized mice was analyzed. The specificity of these antibodies was confirmed by western blotting. However some non-specific bands were observed indicating that some antibodies cross reacted with some *E.coli* proteins. The level of antibodies induced by each vaccine was compared and found that all constructs were capable of inducing a humoral response, however their efficacy was variable. The CMV ie promoter induced the highest antibody level, while the Ad promoters E1A and MLP exhibited lower and almost similar immune responses. These results reflected the

activity of each promoter as seen in our *in vitro* expression results and confirmed the direct correlation between antigen expression and the immune response, lending further support to the accuracy of the *in vitro* results and choice of measure. This relationship was also proven by Galvin *et al.* (2000). They demonstrated that HIV-1 Gag and Env antibody responses, which were induced by the CMV ie promoter, were higher than that of murine leukemia viral long terminal repeat promoters. They also showed that the higher antibody titer was related to the stronger promoter. The relation between the strong promoter and antibody titer is explained by the high antigen expression, which consequently enhances antigen presentation by APCs, and produces a potent immune response. Although Ad promoters were weaker than CMV ie, and exhibited lower antigen expression, they were still capable of inducing antibodies which could be further enhanced by using adjuvants. This may be useful in case of antigens that, if expressed in large amounts, would cause detrimental effects, such as the oncogenic E7 protein of Human papilloma virus (HPV) (Heck *et al.*, 1992). In this study, other promoters were explored particularly, the effect of early and late promoters on the immune response were compared. It was anticipated that using immediate early promoters, even if they are weak, would induce a more rapid immune response and a higher titer of antibodies than strong but late promoters. However, the *in vitro* and *in vivo* results showed that the early promoter did not enhance the expression or the immune response. This indicates that strong promoter like CMV ie is more effective in enhancing gene expressing and also vaccine immunogenicity. However, CMV ie has been used in DNA vaccine trials and could not induce optimal immunogenicity in humans. That underscores the need to explore more powerful approaches to enhance antigen expression. In addition, combining

a strong promoter with codon optimization or a potent adjuvant would optimize the efficacy of DNA vaccines. Enhancing gene uptake is also important and could be realized by using different methods of delivery such as electroporation or gene gun technology that are more effective than i.m. injection and require a lower amount of DNA.

Both antigens E1 and NS3 produce high levels of antibodies under the control of CMV ie, (Figures 2.24, 2.25), which indicates that they could be candidate vaccines that if given together, would maximize protection. However, this study was limited in that only one time point could be evaluated (2 weeks after the last dose). In future experiments the immune response can be monitored over longer periods to investigate the duration of the immune response and indicate the effectiveness of the vaccine. However, this vaccine with some more optimization would be expected to protect against heterologous strains of HCV due to the genetic conservation of E1 and NS3 among strains. However, the effect of DNA vaccine in small animals does not necessarily reflect the response in humans. Therefore, further exploration of these vaccines in large animals would be interesting to investigate their effectiveness for clinical trials.

To conclude, the CMV ie promoter induced an enhanced immune response compared to the Ad promoters, indicating that a stronger promoter is more important for vaccine efficacy than an early promoter. However, since both Ad promoters induced significant level of antibodies, a small amount of DNA is enough to produce an immune response in small animals. That also suggests that any increase in the antigen expression even if small, could effectively enhance the immunogenicity of the DNA vaccine.

Therefore further exploration of different approaches to enhance antigen expression is warranted.

2.4.3- The serum miRNA levels in immunized animals

miRNAs are small players that are involved in most cellular functions. Immunological functions, including immune cell maturation, differentiation and the ability to induce an immune response, are all controlled with a network of miRNA regulators. In this experiment, the relationship between miRNAs and the immune response was investigated by monitoring the change in serum miRNA levels after immunization. Certain miRNAs that are involved in immune system regulation were tested. It has been observed, in Figure 2.28, that mir-181 is up-regulated in all immunized animals compared to the controls, except for one group which was injected with the plasmid pCNS3. mir-21 and mir-296 were up-regulated only in some groups. However these changes were not statistically significant. Connecting these results to the roles of these miRNAs indicates that they are implicated in the regulation of the immune response and also can be indicators for detection of the immune response after vaccination. miRNAs were shown to be regulated through TLR stimulation and activation of the transcription factor nuclear factor-kappa B (NF-κB) pathway. In the case of plasmid DNA, the CpG motifs in the plasmid backbone stimulate the TLR-9 and induce a cascade of pro-inflammatory cytokines that is expected to up-regulate mir-21, mir-181 and mir-196 (Moschos *et al.*, 2007; Sonkoly *et al*., 2008, Marquez *et al*., 2010).

mir-21 has a key role in negative regulation of TLR-dependent inflammatory cytokines to reduce their harmful effect on the host (Sheedy *et al*., 2010). Others reported that mir-21 activates NF-κB increasing the inflammatory response (Shin *et al*., 2011).

Up-regulation of mir-21 in this experiment indicates the activation of the immune system after vaccination by CpG motifs in the plasmid vaccines which are known to activate other pro-inflammatory cytokines. Therefore, whether it acts as a positive or negative regulator of NF-κB, mir-21 appears to be involved in immune response regulation and the increase in its expression reflects the stimulation of cytokines after vaccination.

mir-181 is involved in the activation of B-cells (Chen *et al*; 2004; Shivdasani, 2006) and T-cells, as well as the increase in T cell receptor (TCR) expression. The up-regulation of mir-181 in some vaccinated groups suggests it is vital to the activation of the immune response and may be one of the mechanisms by which DNA vaccine induces its effect. Mir-296 has an antiviral effect and has been shown to inhibit viral replication. Induction of mir-296 by plasmid vaccines is stimulated by IFN-B induced through CpG dependent stimulation of TLR. This confirms that DNA vaccines can have a therapeutic effect not only by priming the immune response but also by induction of anti-HCV miRNAs including mir-196, mir-296, mir-351, mir-431 and mir-448, all of which are induced by IFN-B and inhibit HCV replication (Pedersen *et al.*, 2007).

The variation in the level of the miRNA expression in the vaccinated groups may be a result of the variable number of CpG motifs in different plasmid backbones. Moreover, the expression level does not reflect the antibody level in the different groups, which shows that the immune response is regulated by a complex network of miRNA and other factors. Besides, the miRNAs regulate the immune response in a nonspecific manner via activation of the innate response, while the specific activation of the adaptive immunity by the antigen still plays a major part. Also the variation in miRNAs levels in different animals before immunization might have an impact. Therefore, pre-

immunization sera collection to compare the level of miRNA before and after the vaccination would give more accurate results. Since the changes miRNAs under test were not significant, optimization of the CpG motif content in the DNA vaccine may enhance miRNA expression and hence increase immunogenicity.

In general this study showed that miRNAs are possibly involved in the immune response, suggesting that stimulation of certain miRNAs or co-delivery with a DNA vaccine could enhance the immunogenicity of the vaccine.

2.5- Conclusion

In this study the effect of promoters on the efficacy of DNA vaccines against HCV were evaluated. Plasmids were constructed encoding HCV antigens NS3 and E1 under different promoters. A weak early promoter (E1Ap) and a strong late promoter (MLP) derived from adenovirus, were compared to the standard strong early promoter CMV ie. The effects of these promoters were evaluated based on antibody titers and miRNA expression profile. Based on data reported in this chapter, it is concluded that:

1- The *in vitro* expression by CMV ie is much stronger than Ad promoters.

2- The E1A and Ad MLP promoters showed similar expression pattern in small animals. It would be interesting to see the expression pattern in larger primates.

3- CMV ie induced a higher antibody level after vaccination, indicating that strong early promoters are required for maximum performance of a DNA vaccine.

4- Measuring miRNAs levels, induced after vaccination, showed that plasmid DNA stimulates miRNA induction. Although not in a significant level, these results merit further investigation into the relationship between vaccine CpG content and miRNA level.

Chapter 3

EVALUATION OF ADENOVIRUS AS A RECOMBINANT VACCINE AGAINST HCV

3.1- Introduction

HCV causes a chronic infection in more than 170 million people in different areas of the world. The persistence of the virus is due to an ineffective host immune response. However, it was found that 15% of patients were able to clear the infection in 6 months. Spontaneous viral clearance is related to a potent cellular immune response, which can eliminate the infected cells and completely resolve the virus (Takaki *et al.*, 2000; Vertuani *et al.,* 2002). This finding emphasizes the importance of vaccination as a therapeutic and preventative tool. The DNA vaccine strategy is promising but it shows some limitations in clinical trials. One major factor is limited antigen uptake and amount expressed in host cells after plasmid DNA injection (Manoj *et al.,* 2004). Similar to the DNA vaccine, recombinant viral vectors express the antigen intracellularly and stimulate both the humoral and cellular immune response. However, recombinant viral vectors are more effective in infecting the cell and delivering the antigen encoding gene to the nucleus. Moreover, viral vectors enhance the immunogenicity of the vaccine via stimulation of TLR-dependent innate immunity (Zhu *et al.,* 2007). Human Ads have proven to be effective vectors for delivery and expression (Haj-Ahmad and Graham, 1986; Morin *et al.*, 1987; Schneider *et al.*, 1989) and they have number of advantages. First, they are well characterized and easy to manipulate. Second, they can be grown to high titer. Third, Ad can package 105% of its genome size into its capsid,

accommodating up to 1.8 kb of foreign DNA (Bett *et al*., 1993). Replication defective Ad vector with E1 and E3 deletions can accommodate a larger insert of around 7.5 kb (Xiang *et al*., 1996; Makimura *et al*., 1996). Ad vector vaccines have induced potent neutralizing antibodies and potent $CD8^+$ T cell response against several pathogens including rabies (Xiang *et al*., 1996) and HIV (Shiver *et al*., 2002) and HCV (Arribillaga *et al.*, 2002). Cells infected with Ad vectors live for 7-10 days before they are cleared by CTL in mice. This allows for long-lasting antigen presentation (Yang *et al*., 1994).

Ad activates the innate immune system through several pathways. Viral DNA stimulates the cellular TLR2 and TLR9 receptors in the endosome (Yamaguchi *et al*., 2007; Appledorn *et al*., 2008) and in the cytoplasm via NALT3, a Nucleotide Oligomerization Domain (NOD)-like receptor family member (Muruve *et al*., 2008). Ad signaling results in the activation of the IFN pathway, and secretion of pro-inflammatory cytokines such as IL-6, IL-12, IFN and tumor necrosis factor-α (TNF-α) (Zhang *et al*., 2001). Ad vaccine also causes DC maturation (Molinier-Frenkel *et al*., 2003; Philpott *et al*., 2004), and initiates adaptive immunity against the transgene and Ad genes. The main problem with the Ad vaccine is pre-existing immunity against the virus. Repeated administration of the same viral vector greatly reduces the immune response.

In this study, Ad was evaluated as a vaccine vehicle against HCV. To achieve this purpose, a recombinant Ad vector encoding the NS3 gene (rAdNS3) was constructed and the efficacy of the vaccine was evaluated by measuring the NS3 antibody levels in immunized animals. In addition, the levels of specific miRNAs including mir-181, mir-21 and mir-296 were evaluated in the sera of immunized mice. These miRNAs are involved in immune response regulation.

3.2- Methods

3.2.1- Cell culture

Human embryonic kidney (HEK) 293 cells (Graham *et al*., 1977; Microbix Inc., ATCC CRL-1573) were used for the construction of the adenovirus vectors. The cells were maintained in MEM (Invitrogen) supplemented with 10% (v/v) FBS (Hyclone), 1% (v/v) Antibiotic-Antimycotic (10,000 units/mL penicillin G sodium, 10,000 µg/mL streptomycin sulfate and 25 µg/mL amphotericin B; (Invitrogen Corp., Gibco), 1% GLUTAMAX^{TM}-1 (Invitrogen Corp., Gibco), 3% (v/v) sodium bicarbonate (Invitrogen Corp., Gibco), at 37° C in 5% CO_2. Cells containing the virus were grown in complete MEM supplemented with 5% FBS. The HEK 293 cells were passaged every 3 days at a split ratio of 1:2 after treatment with saline citrate (15 mM sodium citrate, 135 mM potassium chloride).

HeLa cells (ATCC) were used in the evaluation of adenovirus activity. They were maintained similarly to HEK 293 cells, except for the serum used and the cell lifting step. The MEM media was supplemented with 10% Donor Horse Serum (DHS) (PAA Laboratories Inc.) and the cells were detached using 0.25% trypsin (Invitrogen Corp., Gibco) in 4 mL of versene (for 1 L: 1.0g glucose, 0.4g KCl, 8.0g NaCl, 0.58g $NaHCO_3$ and 0.2g EDTA). The HeLa cells were split at a ratio of 1:3.

3.2.2- Adenoviral culture

3.2.2.1- Adenovirus generation

Recombinant adenovirus vectors encoding the NS3 gene were constructed based on the AdMax (Microbix) Ad vector system (Ng *et al*., 1999). This method was based on

homologous recombination between a shuttle vector including the NS3 transcription cassette and Ad vector plasmid. The shuttle plasmid was a modified version of the pDC511 plasmid (Microbix) constructed by inserting an antigen transcription cassette into the plasmid. The shuttle plasmid contains the left end and right end of the Ad5 genome. It also contains an Frt site, which is essential for FLP (a yeast recombinase)-mediated site-specific recombination. The construction of the shuttle vector pDCNS3 was previously shown in Figure 2.12. The Ad vector pBHGfrtΔE1E3FLP contains almost all of the Ad5 genome except for two deletions: E1 and E3 regions. In addition, it contains an FLP gene cassette that expresses the recombinase FLP. This recombinase mediates site-specific recombination at the Frt site. The two plasmids were co-transfected into HEK 293 cells, which produce the E1 protein necessary for viral packaging. The co-transfection was carried out in a 6-well plate. For each well, an aliquot of transfection mixture, containing 5 µL lipofectamine 2000 (Invitrogen) and 2.5 µg from each plasmid in 500 µL Opti-MEM medium (Invitrogen), was used. Once inside the host cell, homologous recombination occurs at the homologous regions shared by the shuttle and genomic plasmid, thus the transgene cassette transferred into the Ad vector. After 10-15 d, the cells were examined for typical adenovirus cytopathic effect (CPE). The resultant viruses are capable of infecting the experimental host cells, but are replication defective due to the absence of the E1 region. The schematic representation of rescuing the virus is illustrated in Figure 3.1.

3.2.2.2- Viral DNA extraction and confirmation

When the cells in the monolayer displayed complete CPE (i.e, become rounded but still attached to the plate), the medium was collected and stored at -70° C after adding

sterile glycerol to a final concentration of 10% v/v. This medium contained released viruses that could be used for preliminary experiments. The infected cells, which remained attached to the surface of the plate, were lysed to extract viral DNA using a modification of the Hirt extraction method (Hirt, 1967). Briefly, 500µL of lysis solution (10 mM Tris-HCl, 100 mM EDTA, 0.4% (w/v) SDS, 0.5 mg/mL pronase) was added to the attached cells and the plate was incubated at 37° C for 4-10 h. The lysate was loaded on a Norgen DNA isolation column to recover the viral DNA. Confirmation of the viral DNA was done by restriction enzyme digestion with *Hin*dIII and gel electrophoresis.

3.2.2.3- High titer adenovirus production

The medium collected from the initial stock was used to infect more HEK 293 cells on 100 mm plates. Two millilitres of virus medium was diluted in a total volume of 8 mL PBS++ infection medium (0.01% $CaCl_2.2H_2O$ and 0.01% $MgCl_2.6H_2O$ dissolved in PBS). The medium was aspirated from HEK 293 cells, and the infection medium was added and incubated for 1 h at 37° C and 5% CO_2. Complete growth medium was added to 20 mL, and incubated. A total of 15 plates of HEK 293 cells with 90% confluence were infected. When the cells displayed complete CPE, the cells were scraped with a rubber policeman. The medium and cells were collected in a 50 cc centrifuge tube and centrifuged at 1000 x g for 5 min. The supernatant was collected and stored at -70° C. The cell pellet was subjected to three rounds of freezing and thawing to release the virus. The crude virus stock was supplemented with 10% sterile glycerol and stored at -70° C.

3.2.2.4- CsCl purification and concentration of Adenovirus

For each 150 mm plate, the medium containing infected cells was collected and centrifuged. The cell pellet was suspended in 1 mL 15 mM Tris, pH 8. The cell suspension was subjected to 4-5 rounds of freezing and thawing to lyse the cells and release the virus. The cell debris was removed by centrifugation at 2000 x g for 5 min. For every 3.2 mL of cell-viral suspension, 1.8 mL of saturated cesium chloride dissolved in 15 mM Tris buffer was added, resulting in a density of 1.35 g/mL. This mixture was aliquoted into the appropriate Beckman ultracentrifuge tubes with a rubber seal lid, and centrifuged at in a type 70.1 Ti rotor in a Beckman L8-80M ultracentrifuge at 246,960 x g for 22 h at 22°C. The viral particles then appeared as a single band in the middle of the tube. The viral band was collected using a syringe with 21 gauge needle after puncturing the tube slightly above the band. The viral bands were pooled in a final volume of 1.5 mL. To get rid of Cscl, the virus stock was dialyzed at 4° C against two changes of 4 L PBS buffer, with a pH of 7.4. The virus was stored at -70° C after adding sterile glycerol to a final concentration of 10%.

3.2.2.5- Adenovirus titration

Adenovirus titration was performed either by RT-qPCR or by measuring the absorbance at 260 nm to determine the number of particles per mL. One A_{260} unit (one O.D. unit) is equivalent to ~10^{12} viral particles (VP)/mL (Maizel *et al.,* 1968). To determine the PFU/mL, a plaque assay was used.

The plaque assay was carried out by infecting HEK 293 cells with serial dilutions of the virus. HEK 293 cells were grown in 6-well plates until 80-90% confluent. Ten-fold dilutions of a viral stock were prepared in 1 mL PBS++. The medium was aspirated,

and a 0.5 mL aliquot of each dilution was added to the cell monolayer in each well, in duplicate. The plate was incubated for 1 h allowing for viral attachment to the cells. The cells in each well were overlaid with a 6 mL mixture of equal volumes of 1% melted agarose solution with 2 x MEM medium, and incubated at 44° C. The overlay was allowed to solidify for 15 min at room temperature before returning the plate to the incubator. The plaques were counted 5-7 d post-infection from dishes bearing approximately 20 to 80 plaques. The viral concentration was determined in PFU/mL as follows: titer = (number of plaques)(dilution factor)/(infection volume).

For isolation of recombinant viruses by plaque purification, well isolated plaques were picked around 5 d post-infection. The plaque-purified vectors were used as inoculum for the preparation of the high-titer viral stocks described previously.

3.2.2.6- Adenovirus infection

Ad infection of mammalian cells was carried out in 6-well plates using a volume of the viral stock equivalent to the desired multiplicity of infection (MOI). This viral volume was mixed with PBS++ in a total volume of 500 µL/ well, and was then added to the cell monolayer (after aspirating the medium). The 6-well plate was then incubated for 1 h at 37º C with 96% relative humidity and 5% CO_2. The plate was swirled every 15 min. Subsequently, 2 mL of the culture medium was added to each well, and the plate was incubated for 24 h.

3.2.3- *In vitro* evaluation of adenoviral transcription activity

rAdNS3 mRNA was quantified using qRT-PCR as explained earlier in Chapter 2**.**

3.2.4- Animals and immunization protocol

Female BALB/c mice aged 6-8 weeks old (18-20 g of weight) were purchased from Charles River Breeding Laboratories, and housed at the animal care facilities of Brock University. All animal work was approved by the Brock University ACC, and done according to CCAC guidelines required for experimentation with animals. Three animals were used for each group. Immunizations with rAdNS3 were accomplished by inoculating 1×10^7 PFU in 100 µL of PBS, into the TA muscle bilaterally. Animals were given only one dose. The control group was injected with PBS. Mice were anaesthetized by inhalation of 5% isoflurane with oxygen prior to injection.

3.2.5- Blood collection and serum preparation

Blood samples were collected by heart puncture 6 wk after immunization. The blood was incubated in an upright position at room temperature for 30-45 min to allow for clotting, and then centrifuged at 4º C for 15 min at 7000 x g. The serum (supernatant) was collected, and small aliquots were stored at -80º C.

3.2.6- Immunological analysis

3.2.6.1- Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was used to monitor the levels of NS3-specific antibodies in the sera collected from immunized animals. ELISA Immulon 4HBX plates (96-well) (VWR International, LLC) were coated with recombinant NS3 (expressed in *E.coli*) at 0.1 µg/well in a bicarbonate buffer (50 mM $NaHCO_3$ buffer, pH 9.6) overnight at 4º C. The plate was washed 5 times with wash solution (PBS + 0.1% Tween 20). The active sites on the plates were blocked with wash solution containing 2% BSA for 2 h at room

temperature to block non-specific antibody binding. The plate was washed again and two-fold serial dilutions of murine sera (in PBS containing 1% BSA) were added to each well, and incubated for 2 h at room temperature on an orbital shaker. To determine IgG levels, 100 µL of peroxidase-conjugated goat anti-mouse IgG antibody (Sigma), at a dilution of 1:5000, was added to each well. The reactions were initiated by adding OPD substrate (Sigma) at 200 µL/well followed by 30 minute incubation at room temperature. The OD was measured at 450 nm by a thermo scientific ELISA reader. OD values had to be at least twice as high as the control values from non- immunized animals to be considered positive.

3.2.6.2- Western blot

Western blots were performed using standard protocols. Briefly, the proteins were separated on a 12% SDS-polyacrylamide gel followed by electrophoretic transfer to a polyvinylidene difluoride (PVDF) membrane (Pall Sciences). The membrane was soaked in methanol, followed by soaking in 1X Blotting Buffer (along with the blotting paper and the SDS-PAGE gel containing the protein). The transfer unit was then assembled according to the manufacturer's instructions, and the proteins were transferred to the PVDF membrane at 40 mA per gel for 1 h. The PVDF was then blocked overnight using 5% Skim Milk 1X TBST (8.8 g of NaCl, 0.2 g of KCl, 3g of Tris base and 500µL of Tween-20 / L, pH 7.4) and incubated with pooled sera from each injected animal group (1:1000 dilution in 0.5% skim milk). The PVDF membrane was then washed three times with 1X TBST, and reacted with 1/5000 dilution of HRP-conjugated anti-mouse IgG (Sigma). After three washing steps, the membrane was developed by chemiluminescent detection using a Pierce CN/DAB Kit (Thermo Fisher Scientific).

3.2.6.3-miRNA quantification

Specific miRNAs involved in the regulation of the immune response were quantified in the serum of immunized and control mice using qRT-PCR according to the standard technique described in Chapter 2 with some modifications. First, total RNA was isolated from 100 µL serum using the Total RNA Purification Kit (Norgen Biotek Corp.) according to the manufacturer's instructions. A 20 µL RT reaction was performed using a 0.5 µL (50 mM stock) stem loop primer specific to each miRNA and 200 ng of RNA.

Real-time PCR was performed on the cDNA using specific forward primers for each miRNA and one universal reverse primer. For relative quantification of miRNA levels, the Ct values were normalized to the 5SrRNA gene. The expression levels of miRNAs were indicated as fold-difference in expression compared to the non-immunized animals. The fold change values were calculated with the ΔΔCt method. Primers used are shown in Table 2.2 (Chapter 2).

3.3-Results

3.3.1- Confirmation of adenovirus encoding HCV-NS3 (rAdNS3)

Adenovirus encoding HCV-NS3 was rescued by homologous recombination between the shuttle plasmid pDCNS3 encoding NS3 under the CMV ie promoter and the genomic adenovirus plasmid pBHGfrtΔE1E3FLP. The resultant virus was designated rAdNS3. To confirm recovery of the appropriate costruct, viral DNA was isolated from the infected HEK 293 cells and digested with *Hin*dIII. Figure 3.1 illustrates the construction of rAdNS3 virus and Figure 3.2 shows the restriction digestion confirmation of rAdNS3 and the expected bands compared to the wild type Ad5. The viral DNA bands appear on a smear background of cellular DNA.

The construct was also confirmed by PCR using two primers, the first primer, for Ad E1 region (AdE1), was used to check for possible contamination by wild type Ad5 that may have developed by homologous recombination with the E1 available in HEK 293 cells. The DNA isolated from infected HEK 293 cells was used as the template. Since the constructed virus has an E1 deletion, the viral DNA showed no amplification. Plasmid DNA containing E1 was used as a positive control. The second primer, the NS3 primer, gave a PCR product of 283 bp as expected for NS3 amplicon, which confirmed the presence of the NS3 gene in the virus. Figure 3.3 shows the PCR product of both primers. After confirmation, the virus was purified by cesium chloride banding and titrated by plaque assay.

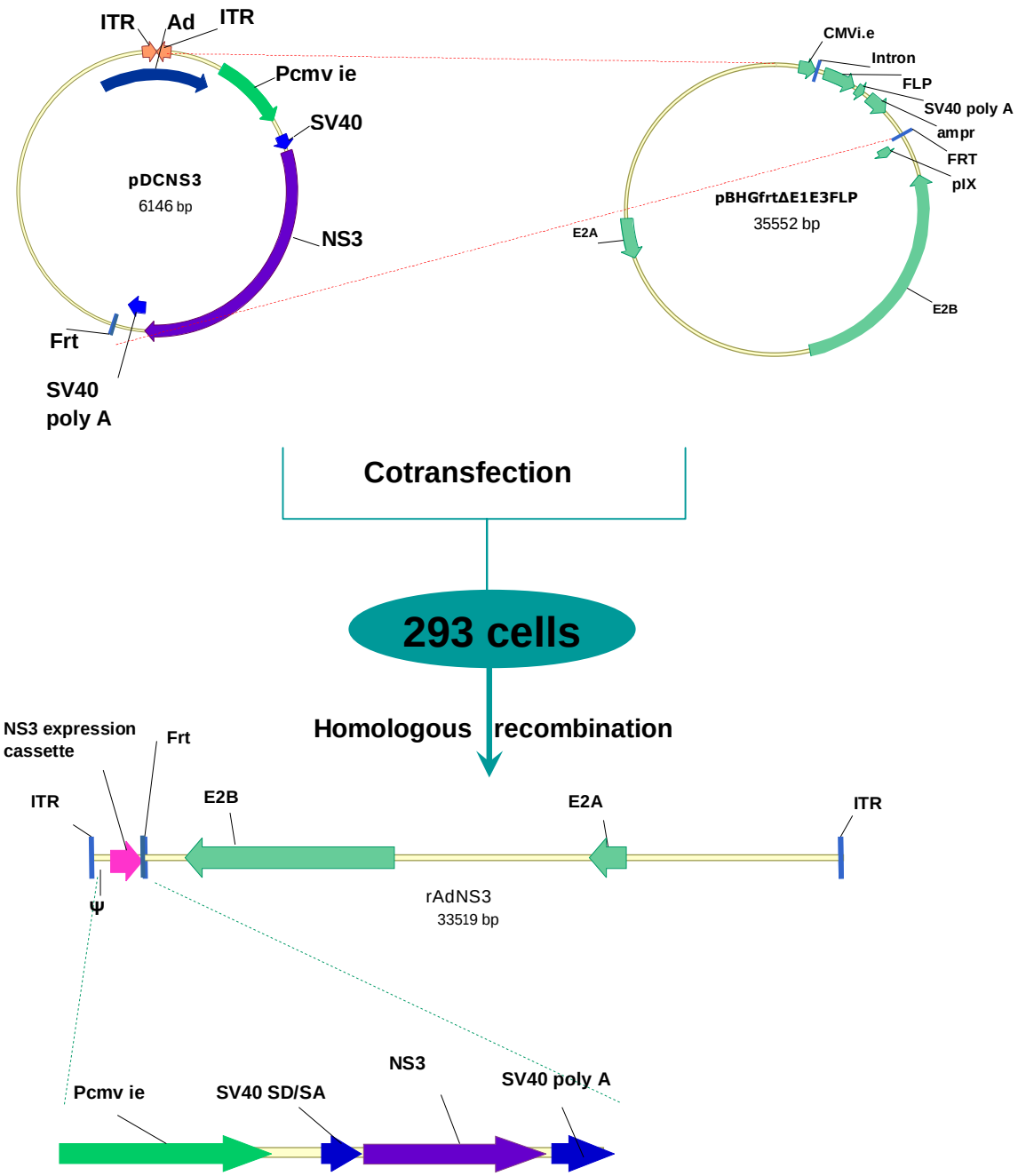


Figure 3.1: Schematic diagram representing the construction of the recombinant adenovirus (rAdNS3) through a homologous recombination system in HEK 293 cells.

Table 3.1: Restriction enzyme analysis of rAdNS3 digested by *Hin*dIII compared to the wild type adenovirus. The fragment sizes underlined indicate the bands that are different from Ad5 and specific to rAdNS3.

***Hin*dIII digestion**	**Wild type Ad5**	**rAdNS3**
Fragments (bp)	**8010** **<u>5665</u>** **5324** **4597** **<u>3437</u>** **2937** **<u>2804</u>** **2081** **1008** **75**	**8010** **<u>6121</u>** **5322** **4597** **<u>3012</u>** **2937** **2081** **1004** **75**

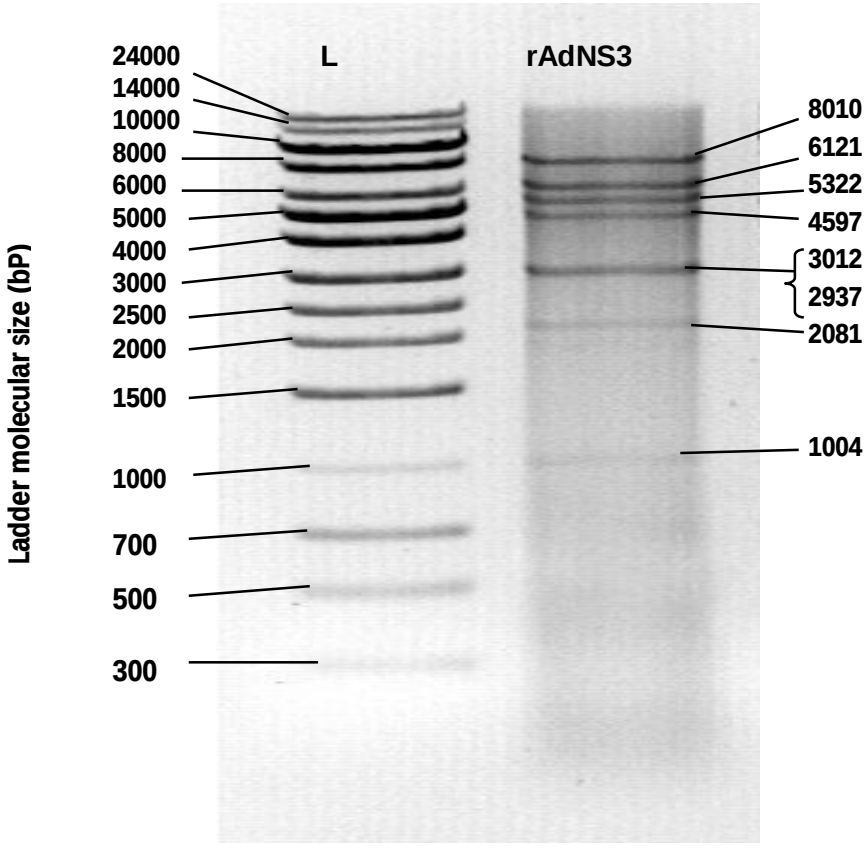


Figure 3.2: Confirmation of rAdNS3 by restriction enzyme digestion. The agarose gel shows the confirmation of rAdNS3 by *Hin*dIII digestion. L is the Norgen's UltraRanger ladder. The size of ladder bands is shown in bp.

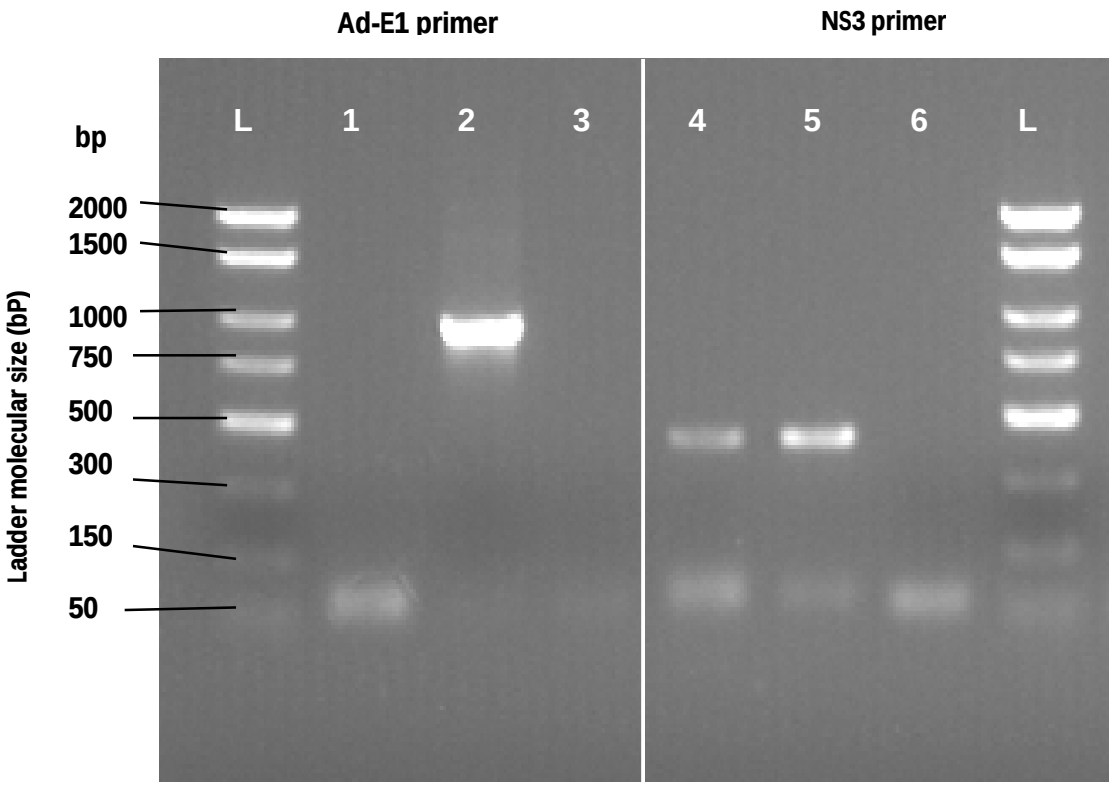


Figure 3.3: Confirmation of rAdNS3 by PCR using DNA isolated from rAdNS3 infected HEK 293 cells. The agarose gel shows amplification using two different primers, Ad-E1 primer: lane 1 (-ve control), lane 2 (+ve control from plasmid encoding E1 produced E1 fragment of 867 bp), lane 3 (rAdNS3 DNA template produced no amplification), and HCV-NS3 primers: lane 4 and 5 (rAdNS3 template produced NS3 band of 396 bp). Lane 6 (-ve control). The first and last lanes are Norgen's FastRunner DNA Ladder. The sizes of ladder bands are shown in bp.

3.3.2- *In-vitro* evaluation of NS3 expression

3.3.2.1- Confirmation of the vaccine's expression activity

The expression activity of the recombinant Ad was confirmed in HeLa cells infected with rAdNS3 at MOI of 5 PFU. The transcription of NS3 was confirmed by RT-PCR 24 h after infection. Figure 3.4 shows the NS3 PCR product with the expected size (396 bp), confirming transcription of the NS3 gene.

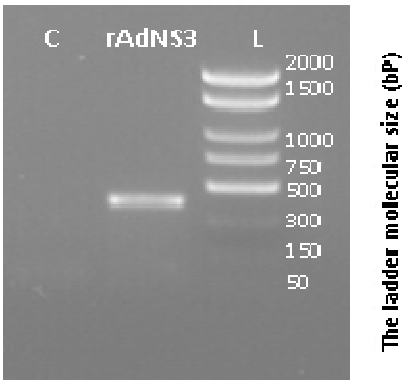


Figure 3.4: Transcription of NS3 mRNA 24 h post-infection of rAdNS3 in HeLa cells. C: the -ve control (no template). rAdNS3: RNA template from infected HeLa cells showed RT-PCR amplification product of NS3 (396 bp). L is the Norgen's FastRunner ladder. The sizes of ladder bands are shown on the right side in bp.

3.3.2.2- Transcription level of HCV- NS3 from adenoviral vectors

HeLa cells were infected with the recombinant adenoviruses rAdNS3 at an MOI of 5. The transcription level of NS3 was evaluated by qRT-PCR at 0, 12, 24 and 48 h after infection. The results showed that adenovirus successfully drove high expression of NS3, represented by high mRNA levels. The mRNA level increased early, at 12 h, and increased at 24 h. It then slightly decreased at 48 h (Figure 3.5).

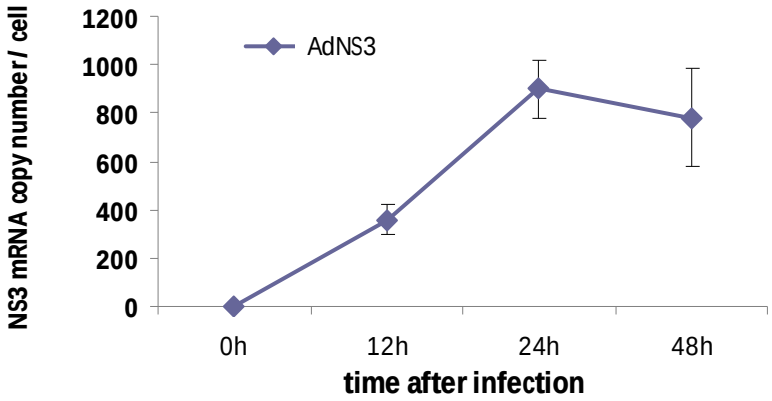


Figure 3.5: The transcription level of NS3 in HeLa cells infected with rAdNS3 over 0, 12, 24, 48h post-infection. Copy numbers were obtained by qPCR using a standard curve of known plasmid DNA concentration. Error bars represents the standard deviation, n=3.

3.3.3- Evaluation of HCV NS3 specific antibody levels in immunized animals

An ELISA was performed to evaluate the antibody against NS3, expressed by the Ad vector, compared to the control animals injected with PBS. The animals showed a high titer of NS3 antibodies six wks after immunization (Figure 3.6). This was higher than antibody level induced 2 weeks after the last boost in the animals injected with the NS3 plasmid vaccine driven with the same promoter (CMV ie) as in the virus (Figure 3.7). The specificity of NS3 antibodies was confirmed by western blot giving signal at the right band size. There were also extra cross reactive bands (Figure 3.8).

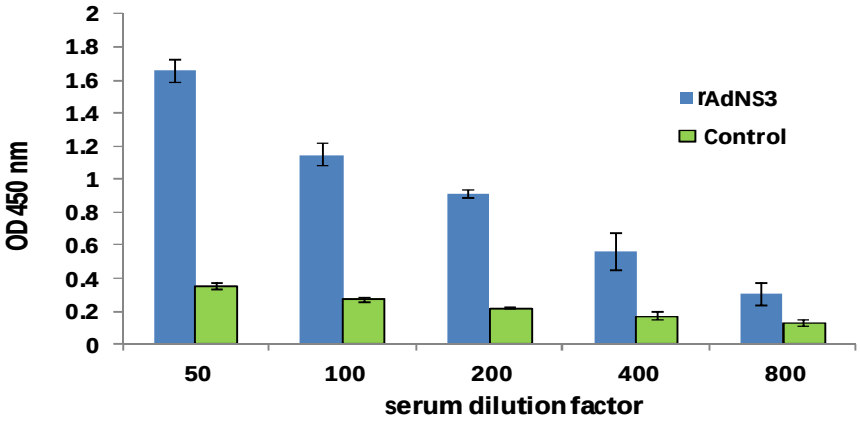


Figure 3.6: NS3 antibody levels induced by the rAdNS3 vaccine 6 weeks after immunization. The antibody levels were measured by OD at 450 nm in different serum dilutions using ELISA. Control animals were injected with PBS. Bars represent the standard deviation, n = 3.

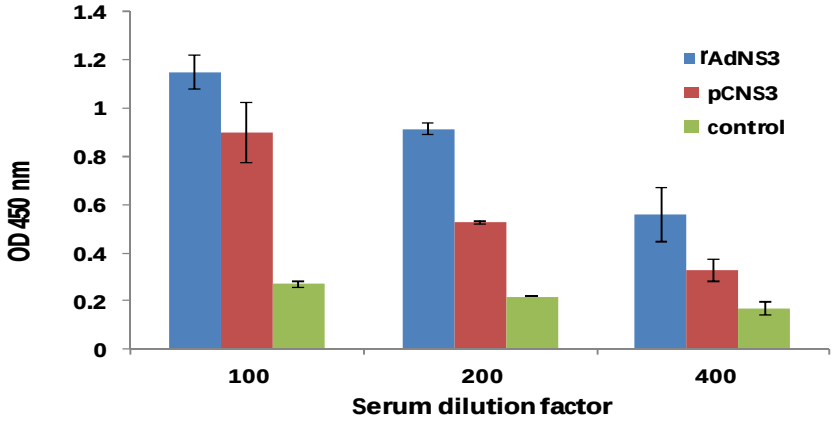


Figure 3.7: Comparison of NS3 antibody levels induced by plasmid (pCNS3) and viral (rAdNS3) vectors. The antibody levels were measured by OD at 450 nm in different serum dilutions using ELISA. Control animals were injected with PBS. Bars represent the standard deviation, n=3.

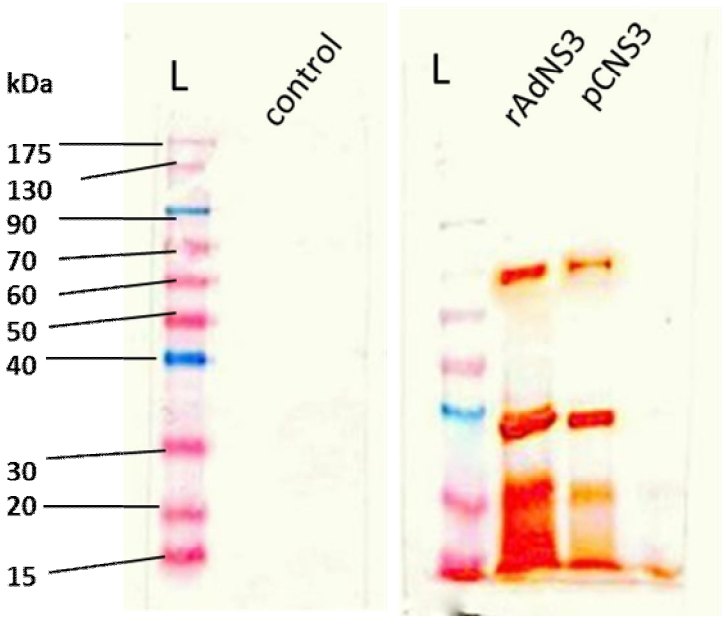


Figure 3.8: Western blot of mice groups immunized with rAdNS3 and pCNS3 compared to non immunized mice. A band around 70 kDa showed the presence of NS3 antibodies**.**

3.3.4- The miRNA level in serum after rAdNS3 immunization

To assess the relationship between the response elicited by immunization and miRNA level, sera were collected from animals injected with pCNS3 and rAdNS3, as well as the control animals (one control had no injection, while the other control was injected with Ad5). qRT-PCR was performed to compare the level of mir-181, mir-21 and mir-296 in all groups. The CT values were normalized to the 5SrRNA gene. Using T-test, the results showed a significant upregulation of mir-181 and mir-21 in animals injected with rAdNS3 and Ad5 ($p < 0.05$). Mir-296 showed higher expression only in animals injected with Ad5 however it was not statistically significant ($p > 0.05$). pCNS3 showed non-significant change as compared to non-immunized control animals ($p > 0.05$). The results are shown in Figure 3.9.

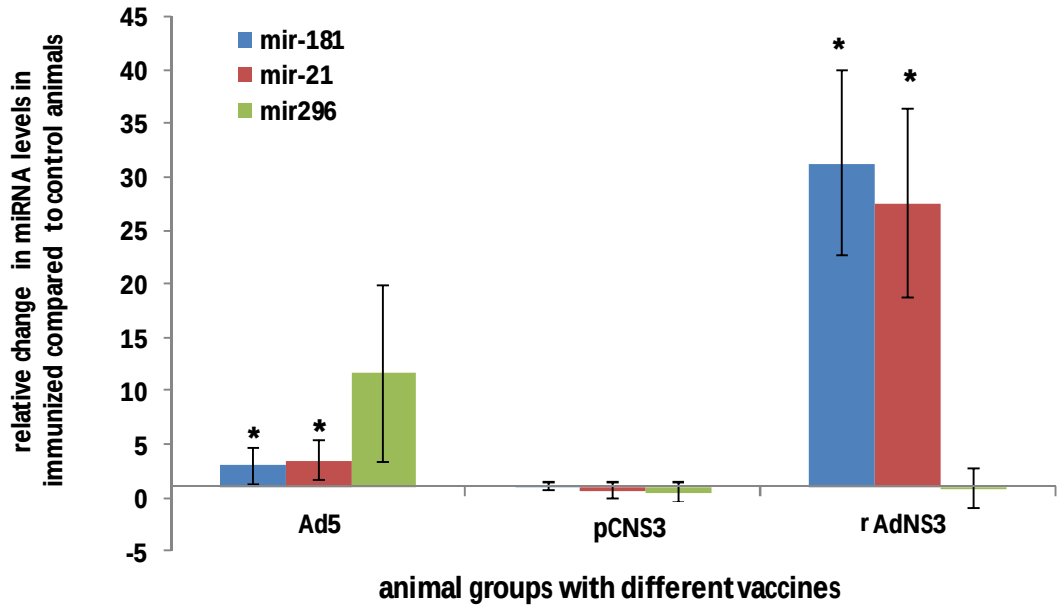


Figure 3.9: The expression levels of microRNAs in the sera of mice immunized with rAdNS3 and pCNS3. The level of mir-181, mir-21 and mir-296 are expressed as a change in fold compared to the levels found in the sera of the control animals (injected with saline) measured by ΔΔCt method. Error bars represent thestandard deviations. n=3. Result is considered significant (*) if p value < 0.05.

3.4- Discussion

HCV clearance requires a potent immune response, including specific antibodies and cytotoxic T cells. Recombinant viral vaccines are an attractive approach to induce strong humoral and cellular immune responses, since both are critical factors against viral infection. Clinical trials have shown that antigen uptake and level of expression are critical factors affecting the efficiency of the vaccine (Manoj *et al.,* 2004). Since antigen expression level correlates with efficient antigen delivery, the delivery vehicle is important in protecting the DNA and enhancing delivery, thus increasing the amount available for expression. Recombinant viral vectors are more efficient in the entry to the host cell via receptor attachment and deliver the DNA to the nucleus more efficiently, being protected by the protein capsid (Miazawa *et al*., 1999). Therefore, viral vectors are good candidates for vaccine delivery. Particularly, Ad is known for its efficient transduction for most types of cells, including APCs, and its high transgene expression capacity.

In this study, an HCV vaccine was constructed based on an Ad vector encoding NS3 (rAdNS3). The Ad vector was evaluated for *in vitro* expression and *in vivo* induction of immune response. Results revealed a high level of NS3 transcription in HeLa cells, as evidenced by the NS3 mRNA level. The mRNA level became detectable at 12 h post-infection and increased with time. We did not test earlier points, however Ad has been shown to express the early genes just 4 h post-infection (Shaw and Ziff, 1982). The high level of NS3 mRNA indicates highly efficient cell infection by the Ad vector. In addition, NS3 was cloned under the control of the potent early CMV ie promoter that provides a high transcription level (Candolfi *et al.,* 2006).

3.4.1- The immune response against rAdNS3 immunization

To evaluate the activity of rAdNS3 vaccine *in vivo*, mice were immunized with rAdNS3 and compared the NS3 antibody level to the plasmid vaccine pCNS3 (Chapter 2). The rAdNS3 vaccine was given in a single dose, as the adenovirus proteins would induce neutralizing antibodies that would inhibit the infection of any subsequent dose, resulting in a decreased immune response (Capone *et al.*, 2006). However, the DNA vaccine requires boosting doses due to the inefficient uptake by APCs. The results showed that rAdNS3 induced higher antibody levels six wks after a single injection, than that elicited two wks after the third boost of pCNS3 (Figure 3.7). Since NS3 transcription is driven with the same promoter (CMV ie) in both vectors, the increase in the immune response is most likely due to the delivery system. This outcome reflects a strong and prolonged immune response elicited by Ad vectors. This response may be driven by various mechanisms including efficient cell transduction, high level antigen expression, and the adjuvant effect of other Ad proteins. These results confirmed the importance of efficient antigen delivery to induce a strong immune response and the advantages of Ad as a vaccine vector. The results are consistent with another report by Arribillaga *et al.* (2002). They showed a potent immune response elicited 10 days after intrapretoneal injection (i.p.) of 10^9 PFU of Adenoviral vector encoding NS3. In the current study, lower dose was used for i.m. injection (10^7 PFU) and immune response was shown for longer peiod (6 wks). Our results indicate that i.m. injection may be more effective than i.p. injection. Prolonged and strong immune responses were also shown by Adenovirus encoding NS3 fused to MHC-II (Mikkelsen *et al.*, 2011).

Since HCV chronicity is caused by a low, ineffective immune response (Thimme *et al.*, 2002), rAdNS3 may be a good candidate as a prophylactic and therapeutic vaccine based on its efficient prolonged immune response. This vaccine warrants further investigation in large animals. Ad vector was tested only with NS3 antigen. However, as shown in Chapter 2, E1 produced a high level of E1 antibodies. Therefore, including E1 antigen in the same Ad vector would be expected to broaden the immune response, inducing neutralizing antibodies and cellular immunity.

3.4.2- The Effect of rAdNS3 vaccination on miRNA expression

To investigate rAdNS3 vaccine activity, and the mechanism of activating innate immunity, the level of selected miRNAs was evaluated in the serum of the immunized animals to monitor any changes compared to control animals. mir-181 and mir-21 were chosen for their role in immune cell differentiation and immune response regulation. Other miRNAs which target the HCV genome and are induced by IFN-β, such as mir-296, were also selected (Pedersen, *et al*., 2007). Comparing the miRNA levels in groups injected with rAdNS3 and pCNS3 to the control animals (Figure 3.9), showed that the animals immunized with rAdNS3 exhibited significantly higher levels of mir-181 and mir-21 than animals injected with pCNS3 and the control animals, while mir-296 did not change with either vector. Since pCNS3 did not induce significant changes in mir-181 and mir-21 levels compared to the control, it is suggested that the change is due to the Ad vector elements rather than the antigen. The higher level of miRNA induced by an Ad vaccine seems to be correlated to the efficient antigen delivery and activation of the innate immune system. miRNAs are induced through TLR activation by dsDNA, as is the case for Ad vectors. These results are in accordance with the finding that miRNAs are

upregulated with viral stimuli through TLR and inflammatory cytokines such as IFN (Sonkoly *et al*., 2008). miRNA upregulation does not seem to be related to the antigen, but to the vector itself. To verify this idea, the miRNA level was compared in animals injected with either rAdNS3 or wild type Ad5 that contains the full adenovirus genome and no antigen insert. Surprisingly, Ad5 was found to induce lower levels of mir-181 and mir-21, and higher levels of mir-296. These observations could be explained by the presence of the E1 and E3 genes in the wild type (Ad5). E1 and E3 are known to subvert the innate immune system, and inhibit the cytokine induction pathways (Russell, 2000; Horwitz, 2001). This is expected to subsequently downregulate mir-181 and mir-21. On the other hand, mir-296 levels were upregulated with Ad5, which is related to the transformation induced by the E1 region. These results indicate that the differential expression of miRNAs is related to Ad vector (rAdNS3) lacking E1 and E3, and those miRNAs are related to the immune response activation. mir-181, reported to be localized in B-lymphocytes, has been implicated in the regulation of the maturation, proliferation, differentiation, and activation of immune cells (Chen *et al*., 2004; Neilson *et al*., 2007; Tsitsiou and Lindsay 2009). It also regulates T-cell receptor signaling (Li *et al*., 2007; Pedersen and David 2008). This is consistent with the high immunogenicity of rAdNS3, and the high antibody induction which resulted from the activation of B-cells induced by mir-181. mir-181 and mir-21 play a role in the regulation of inflammatory cytokine production (Moschos *et al*., 2007). Thus, increasing these miRNAs in immunized animals indicated that cytokine induction by the Ad vector has a role in activation of the immune response. These results confirmed the efficiency of Ad vector for vaccination.

To our knowledge, this is the first study to monitor the change in miRNA levels after vaccination. This study demonstrated a relationship between vaccines and miRNA, suggesting that miRNAs are involved in the regulation of the immune response. Further investigation to understand this relationship will help in the design of an effective therapeutic vaccine against HCV.

3.5-Conclusion

In this chapter, a recombinant Ad expressing the HCV NS3 was constructed and evaluated for its efficiency in immune response induction.

The data presented in this chapter can be summarized as follows:

1- rAdNS3 induced high antibody levels 6 weeks after vaccination with a single dose.

2- rAdNS3 was more efficient than the plasmid vaccine pCNS3.

3- The immune response induced by rAdNS3 correlated with upregulated miRNAs levels, specifically mir-181 and mir-21, which possibly enhance the immune response through the maturation of B and T cells.

CHAPTER 4

ADENOVIRUS MEDIATED-RNA INTERFERENCE AGAINST NS3 REGION OF HEPATITIS C VIRUS

4.1- Introduction

HCV is a global health burden, and it appears to be the main reason for liver transplantation and liver-related mortality. The current standard therapy is a combination of pegylated IFN-α and the nucleotide analogue ribavirin, which induces a sustained virologic response (SVR) in 50% of patients, depending on the virus genotype (Munir *et al.*, 2010; Tsubota *et al.*, 2011). Other factors, such as viral load and the age of the patient, also affect the outcome of the therapy (Foster, 2004; Feld and Hoofnagle, 2005). In addition, IFN/ribavirin is a long-term therapy and causes severe side effects. Therefore, new approaches are now being developed. Recently, RNAi technology attracted attention as a promising antiviral therapy that can suppress viral enzymes involved in replication. RNAi is a process of post-transcriptional gene silencing by a dsRNA of homologous sequence. Long dsRNAs are cleaved by Dicer, an RNase III-like enzyme, into 21-28 bp duplex siRNA (Bernstein *et al.*, 2001; Paddison *et al*., 2002). siRNAs are then incorporated into RISC; the latter guides siRNA to the complementary mRNA, thus leading to the target mRNA cleavage and subsequent degradation with RNase H (Hammond *et al*., 2000; Gregory *et al*., 2005; Liu *et al*., 2004). Alternatively, partial complementarity with the target mRNA would lead to inhibition of protein translation due to steric hindrance (Bartel, 2004, Meister *et al*., 2004). The main factors influencing successful RNAi therapy include target specificity and the effectiveness of

the delivery method. Conserved regions of the virus, particularly with a high mutation-prone virus like HCV, should be targeted, provided that they are crucial for viral replication. The non-structural proteins perform vital processes required for viral replication. The main functional proteins are the protease and helicase encoded by NS3, and RdRP encoded by NS5B, therefore they are good target candidates for the siRNA, new therapeutic approaches.

RNAi has been used for inhibiting several viruses, including HIV (Jacque *et al.*, 2002), poliovirus (Saleh *et al.*, 2004), and HCV (Wilson *et al.*, 2003; Randall *et al*., 2003). Different target regions in the mRNA as well as different siRNA delivery methods were used. RNAi can be induced using siRNA duplex which binds directly to RISC or as shRNA expressed by a plasmid vector, and processed similarly to internal miRNA. The main hurdle to siRNA treatment remains the inefficient transfection method. The low cell transfection efficiency and the susceptibility of small RNAs to degradation by cellular RNases led to the search for an alternative approach. Different transfection methods were used, such as lipofictamine 2000 or electroporation (Wilson *et al*., 2003; Randall *et al*., 2003), however these methods have cytotoxic effects on the cells. Therefore shRNA vectors are preferable methods of delivery. Plasmids expressing shRNAs encoded by plasmid DNA or recombinant lentivirus vector effectively inhibited HCV replication in HCV subgenomic cell lines (Takigawa *et al*., 2004; Eisa *et al.*, 2010). However, plasmid uptake is not effective in humans, resulting in a short duration of gene silencing. Therefore, viral vectors would be more effective. In this study, the use of Ad vector was used to overcome the reported drawback of other delivery methods of siRNA against HCV. Ad was used extensively in transgene expression, due to a number of

advantages including easy manipulation and high titer production. In addition, its wide tropism allows for efficient infection of a variety of cells, including liver cells. Adenovirus also elicits long-lived transgene expression that induces a durable effect. shRNA expressing Ad vectors were used successfully for silencing hepatitis B virus (HBV) (Carmona *et al*., 2006; Uprichard *et al*., 2005) and HCV (Sakamooto *et al*., 2008). Ad has been also used indirectly against HCV by silencing cellular cofactors that support HCV replication (Zhang *et al.*, 2004). In this study, recombinant Ad vectors expressing shRNAs (rAd-shRNAs) against the HCV-NS3 region were constructed. Two different target sites were chosen: one in the protease and one in the helicase encoding sequences. In addition, two different promoters were used, U6 promoter and CMV ie promoter. NS3 gene silencing by rAd-shRNAs was evaluated in Con1/FL-Neo cells.

4.2- Methods

4.2.1- shRNA plasmid construction

4.2.1.1- shRNA sequence synthesis

The shRNA sequence was generated as two complementary oligonucleotides (ODN) (Sigma). Each strand consisted of two complementary sequences separated by a loop. The two strands were annealed and ligated to a shuttle plasmid. Two shRNA sequences were synthesized targeting the NS3 region. The siRNA target sequences were chosen using Ambion's siRNA design tools. The NS3Si-Pro sequence targeted the CCAGGACCUCGUCGGCUGG site, located at 3656-3674 bps of the HCV-1b genome in the protease encoding part of NS3. *Sac*I and *Eco*RI sites were added to the 5' and 3' end of each strand respectively, for cloning in the plasmid pDCG under control of CMV ie promoter. *Sac*I lies in the last part of CMV ie promoter in pDCG plasmid, So this sequence was added to the shRNA oligo to complete the promoter. *Sac*I was used to remove extra sequence between the promoter and the ShRNA. Also poly A signal AATAAA was added at the end of ShRNA sequence (Song *et al*., 2004). The *Avr*II site was added to the 5' end for screening. NS3Si-Hel targeted the CUAGCGGAGACGUCAUUGU site located at 4624– 4642 bps of the HCV genome in the helicase encoding part of NS3. The two strands were synthesized to have *EcoR*I and *Pst*I sites on both ends for cloning under the U6 promoter in the pDU6 plasmid. The *Kpn*I restriction site was added for screening. To synthesize each shRNA, the two ODNs were mixed in an equal molar ratio in 20 μL nuclease free water and annealed using the iCycler PCR machine (Bio-Rad). The ODN mixture was heated to 94° C, followed by

lowering the temperature in decrements of 0.5° C every 10 sec. When the temperature reached 18° C, the mixture was cooled to 4° C. The ODNs used are listed in Table 4.1.

Table 4.1: Oligonucleotides used to generate shRNA against NS3.

shRNA	Oligo	Sequence
shRNA-NS3 protease	FwNS3si-1b	5'CGTTTAGTGAACCGTGGTACCCCAGGACCTCGTCGGCTGGTTCAAGAG ACCAGCCGACGAGGTCCTGGAATAAACCTAGG 3'
	RvNS3si-1b	5'AATTCCTAGGTTTATTCCAGGACCTCGTCGGCTGGTCTCTTGAACCAGC CGACGAGGTCCTGGGGTACCACGGTTCACTAAACGAGCT 3'
shRNA-NS3 helicase	FwNS31b-siU6	5'AATTCTAGCGGAGACGTCATTGTTTCAAGAGAACAATGACGTCTCCGCT AGTTTTTTGGTACCTGCA 3'
	RvNS31b-siU6	5'GGTACCAAAAAACTAGCGGAGACGTCATTGTTCTCTTGAAACAATGACG TCTCCGCTAG 3'

4.2.1.2- shRNA cloning

Plasmids pDCG containing CMV ie promoter and pU6 containing U6 promoters were used to clone the shRNA ODNs resulting in two plasmids, pNS3-Si-Pro and pNS3-Si-Hel respectively. The pDCG plasmid was digested with *Sac*I and *Mfe*I which is compatible with *Eco*RI in shRNA ODN, So EcoRi can be used for screening for the right plasmid. pU6 was digested with *Eco*RI and *Pst*I (NEB).Approximately 0.5-5 µg DNA was digested with 1-10 units of the restriction enzyme, and incubated at 37º C for 2-3 h. Equal amounts of the two restriction enzymes were used with the appropriate buffer recommended by the manufacturer for the combination of these enzymes. The enzymes were then inactivated prior to ligation by heating to 70° C for 20 min. Plasmid and shRNA fragments were ligated at a ratio of 1:3 using 400 units of bacteriophage T4 ligase (NEB) in a 20 µL reaction. The reaction was incubated at room temperature for 2 h. The plasmid was transformed in 100 µL of competent *E. coli* according to standard protocol (Sambrook *et al*., 1989). The transformed cells were spread on a selective agar

plate with ampicillin, and incubated at 37º C for 16 h. A few colonies were picked and cultured overnight at 37º C. The plasmids were isolated and screened via restriction enzyme digestion.

4.2.1.3- Plasmid DNA isolation

Small-scale plasmid DNA isolation was carried out with the Plasmid MiniPrep DNA Kit (Norgen Biotek Corp., ON) according to the manufacturer's instructions. Prior to plasmid transfection, all plasmids were prepared in large scale, and purified with the Endotoxin Free Plasmid DNA MaxiPrep Kit (Norgen Biotek Corp.) according to the manufacturer's instructions.

4.2.1.4- Plasmid confirmation by sequencing

The plasmids were sequenced to confirm the presence of shRNA, and to ensure mutations did not take place. DNA sequencing was performed at York University Core Molecular Biology and DNA Sequencing Facility. Cycle sequencing reactions were carried out with Bigdye Terminator chemistry on the Applied Biosystems 337 DNA Sequencer, using the appropriate primers.

4.2.2- Adenovirus culture

4.2.2.1- Adenoviral vector construction

Recombinant adenovirus vectors encoding shRNAs were constructed by homologous recombination based on the AdMax (Microbix) adenoviral vector system as previously shown in Chapter 3. The shuttle plasmid (pNS3si-Pro or pNS3si-Hel) and the

adenovirus genomic plasmid pBHGfrtΔE1E3FLP were co-transfected into 293 cells, which produce the E1 protein necessary for viral packaging. The co-transfection was carried out in a 6-well plate. For each well, a transfection mixture, containing 5 µL lipofectamine 2000 (Invitrogen) and 2.5 µg from each plasmid in 500 µL Opti-MEM medium (Invitrogen), was used. Complete CPE was observed after 10 days. The medium and infected cells were harvested. To confirm the virus, the cells were lysed to extract viral DNA using a modification of the Hirt extraction method (Hirt, 1967). Briefly, 500µL of lysis solution (10 mM Tris-HCl, 100 mM EDTA, 0.4% (w/v) SDS, 0.5 mg/mL pronase) was added to the attached cells and the plate was then incubated at 37° C for 4-10 h. The lysate was then loaded on a Norgen DNA isolation column to isolate the viral DNA. Confirmation of the viral DNA was done by restriction enzyme digestion with HindIII. Finally the rAd-shRNA vectors were grown, purified on CsCl gradient and titrated using plaque assay.

4.2.2.2- Titration of rAd-shRNA by plaque assay

The plaque assay was performed to quantify the infectious particles of the virus. Briefly, 10-fold dilutions of the virus were prepared in 1 mL PBS++. In a 6-well plate, 80-90% confluent 293 cells were infected with 1 mL of each dilution. After 1 h incubation at 37º C, the cells in each well were covered with a 6 mL mixture of equal volumes of 1% melted agarose solution with 2X MEM medium, at 44°C. The cells were incubated again at 37º C, 5% CO_2 after the agarose layer solidified. The plaques were counted 5-7 d post-infection. The virus concentration was determined in PFU/mL as follows: titer = (number of plaques) (dilution factor)/(infection volume).

4.2.2.3- Adenovirus infection

Con1/FL-Neo cells were infected with MOI 5 of rAd-shRNA targeting NS3. The Ad infection of mammalian cells was carried out in 6-well plates. The virus was mixed with PBS++ in a total volume of 500 µL/ well and then added to the cell monolayer (after aspirating the medium). The 6-well plate was then incubated for 1 h at 37º C with 96% relative humidity and 5% CO_2 with swirling the plate every 15 min. After that, 2 mL of the culture medium was added to each well, and the plate was incubated for 48 h or as needed for the experiment.

4.2.3- Quantification of NS3 inhibition

4.2.3.1- RNA isolation

Total RNA was isolated from FL-Neo con1 cells after transfection with Ad-shRNA. RNA isolation was performed using the Total RNA Purification Kit (Norgen Biotek Corp.) according to the manufacturer's instructions. RNA integrity was assessed by electrophoresis on a formaldehyde agarose gel (Sambrook *et al.* (1989). The gel was viewed and photographed under UV light using the AlphaImager 2200 (Alpha Innotech).

RNA was quantified by spectrophotometry (Sambrook *et al.*, 1989). To inhibit the interference of cellular DNA, 50 µL of the RNA sample was digested with 4 units of TURBO DNaseI (Ambion) in a 100 µL reaction mixture, and incubated at 37° C for 30 min, followed by purification using the CleanUp and Concentration Kit (Norgen Biotek Corp.), according to the manufacturer's instructions.

4.2.3.2- Reverse transcription

Two hundred nanograms of total RNA was used in RT reactions. RNA was combined with 0.5 µL of NS3-specific RT primer (50 mM stock) and completed to 5 µL final volume with RNase/DNase-free water (Ambion). This mixture was incubated for 5 min at 70° C, and then cooled at 4° C. Fifteen microliters of the RT reaction solution was added to the mixture. The RT reaction solution contained 4 µL of 5X First Strand Buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl and 15 mM $MgCl_2$), 2 µL of 0.1 M Dithiothreitol (DTT), 1 µL of 10 mM dNTPs, 0.1 µL Superscript III reverse transcriptase (Invitrogen) and 7.9 µL RNase/DNase-free water. The reaction was incubated at 25° C for 5 min, followed by 90 min at 50° C. The reaction was inactivated by heating at 70° C for 15 min and held at 4° C.

4.2.3.3- Quantitative PCR

The level of NS3 cDNA was quantified by qPCR. The reaction was performed using the Bio-Rad iCycler thermal cycler. In a 20 µL reaction, 10 µL of 2 X SYBR® Green PCR Mastermix (BioRad) was mixed with 1 µL of each primer (5mM stock) and 4 µL of cDNA template. The reaction began with 15 min at 95° C followed by 40 cycles of three steps: 95° C for 15 sec, 60° C for 30 sec and 72° C for 30 sec. The reaction was incubated for 1 min at 57° C before starting a melting curve analysis by a 0.5° C increment decrease every 10 sec over 80 rounds. To determine the initial concentration of NS3 cDNA, the CT values of NS3 were normalized to the house keeping gene S15. The results were calculated with the ΔΔCT method and are indicated as a change in fold compared to the NS3 cDNA levels from non-infected cells.

4.3- Results

4.3.1- Confirmation of the pNS3-Si-Pro plasmid

pNS3-Si-Pro construction and screening is illustrated in Figure 4.1 and 4.2. The plasmid was confirmed with *Nco*I and *Avr*II enzyme digestion and yielded two expected bands, 3638 and 283 bp. The extra band of 2000 bp is possibly due to non-digested plasmid. The plasmid was also confirmed by sequencing.

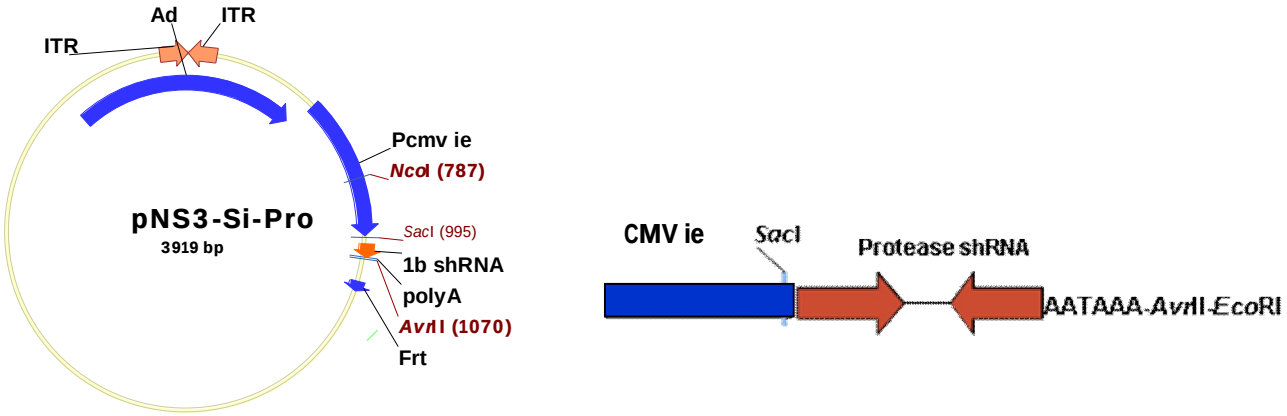


Figure 4.1: Schematic diagram of the construction of the shRNA plasmid against the protease (pNS3-Si-Pro).

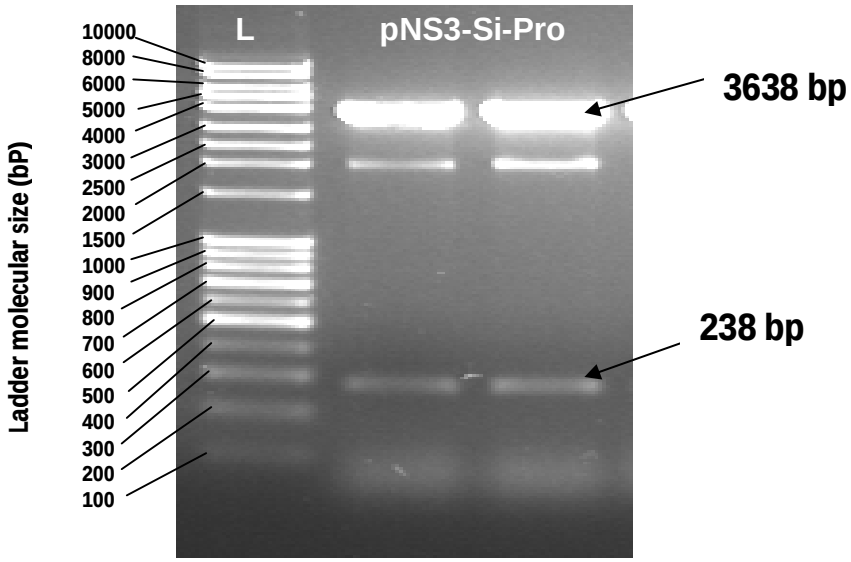


Figure 4.2: Confirmation of pNS3-Si-Pro by restriction digestion. Plasmid DNA was digested with *Nco*I and *Avr*II. The resultant bands were the expected size 3638 and 283 bps. L: Norgen's FullRanger ladder. The size of Ladder bands is shown in bp.

4.3.2- Cconfirmation of the pNS3-Si-Hel plasmid

pNS3-Si-Hel construction and confirmation is illustrated in Figure 4.3 and 4.4. The plasmid was confirmed with *Kpn*I restriction enzyme digestion and yielded two expected bands, 4596 and 757 bp. The plasmid was also confirmed by sequencing.

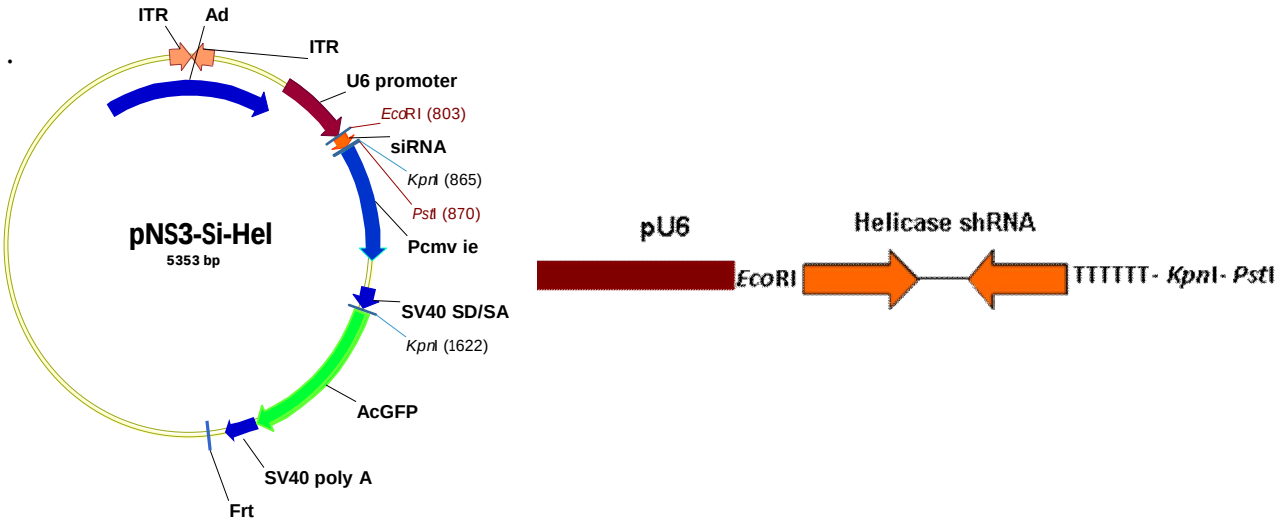


Figure 4.3: Schematic diagram of the construction of shRNA plasmid against helicase (pNS3-Si-Hel).

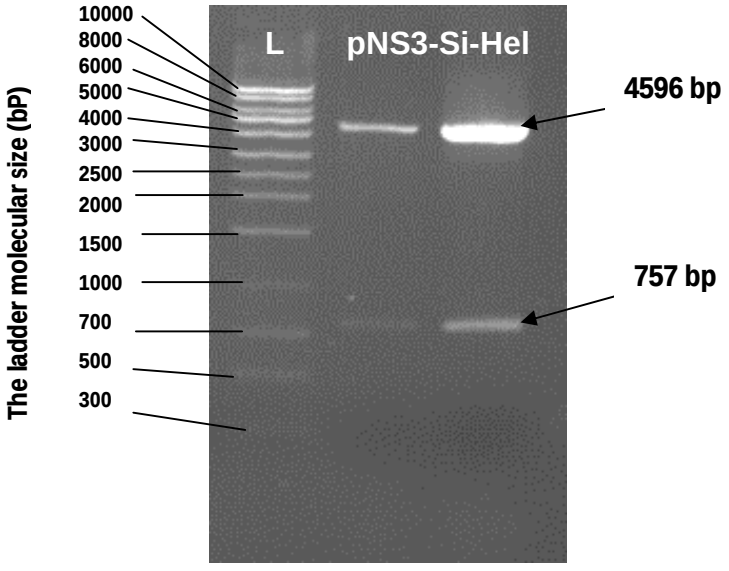


Figure 4.4: Confirmation of pNS3-Si-Hel by restriction enzyme digestion. Plasmid DNA was digested with *Kpn*I. The resultant bands were the expected sizes, 4596 and 757 bps. L: Norgen's MidRanger ladder. The size of ladder bands is shown in bp.

4.3.3- Confirmation of rAd-shRNAs against NS3

Ad vectors encoding shRNA sequences targeted to HCV RNA were rescued by homologous recombination between the shuttle vectors constructed previously (pNS3-Si-Pro or pNS3-Si-Hel) and the Ad plasmid pBHGfrtdel13FLP in 293 cells. The construction strategy is shown in Figures 4.5 and 4.6.

The recombinant viral vectors, rAdsiNS3-Pro and rAdsiNS3-Hel, were confirmed by *Hin*dIII digestion as shown in Figure 4.7. The digestion bands shown are similar to the expected bands from the restriction analysis of the Ad vectors, compared to the wild type virus (Ad5) shown inTable 4.2.

We further confirmed the absence of any wild type virus (Ad5) contamination due to homologous recombination with the Ad E1 gene in 293 cells. PCR was performed on the viral DNA isolated from 293 cells infected with the adenoviral vectors rAdNS3-Si-Pro and rAdNS3-Si-Hel using the AdE1 primer. The PCR results showed no amplification in either viral DNA, confirming that the viruses are resultant only from homologous recombination between the adenovirus plasmid and the shRNA transcription cassette. Plasmid encoding AdE1 (pE1) was used as a positive control template to confirm that PCR reaction worked. The PCR results are shown in Figure 4.8.

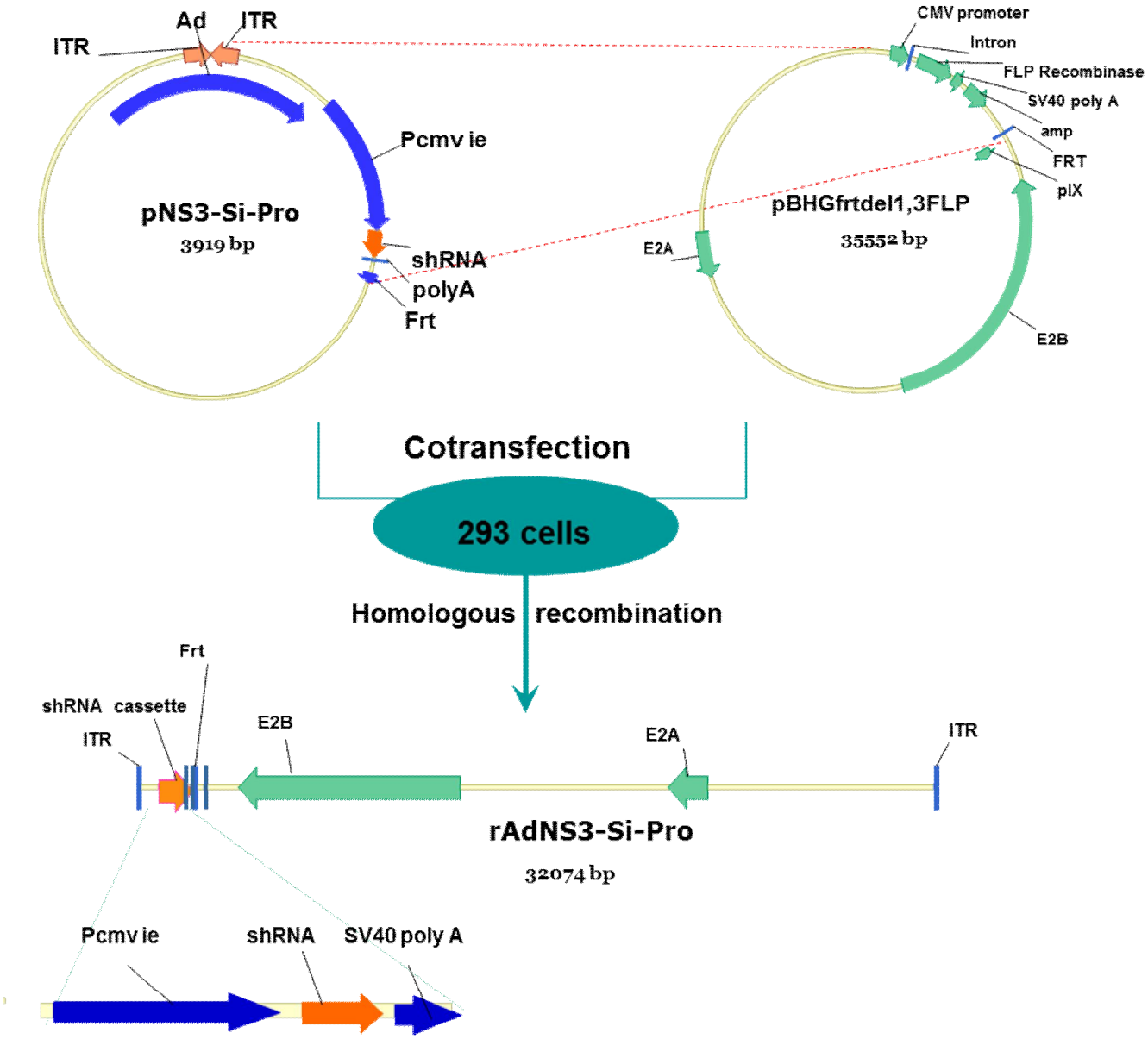


Figure 4.5: Schematic diagram representing the construction of the recombinant adenovirus (rAdNS3-Si-Pro) through a homologous recombination system in HEK 293 cells.

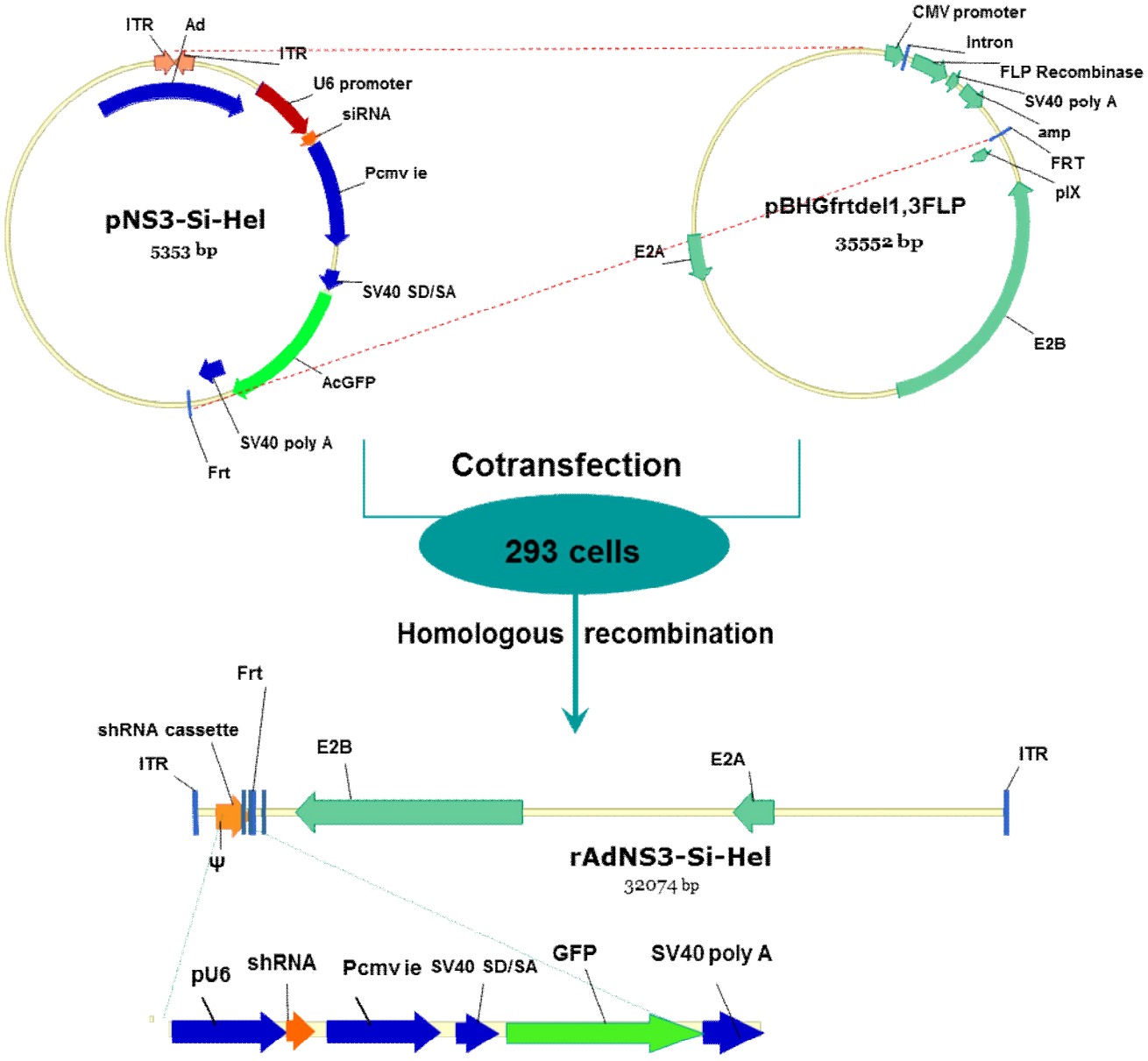


Figure 4.6: Schematic diagram representing the construction of the recombinant adenovirus (rAdNS3-Si-Hel) through a homologous recombination system in HEK 293 cells.

Table 4.2: Restriction enzyme analysis of Ad-shRNAs against NS3 and Ad5. The fragment sizes underlined indicate the bands that are different from Ad5 and specific to rAd-shRNAs.

***Hin*dIII digestion**	**Wild type Ad5**	**rAdNS3-Si-Pro**	**rAdNS3-Si-Hel**
Fragments (bp)	**8010** **<u>5665</u>** **5324** **4597** **<u>3437</u>** **2937** **<u>2804</u>** **2081** **1008** **75**	**8010** **5322** **4597** **<u>3894</u>** **<u>3012</u>** **2937** **2081** **1004** **75**	**8010** **<u>5328</u>** **5322** **4597** **<u>3012</u>** **2937** **2081** **1004** **75**

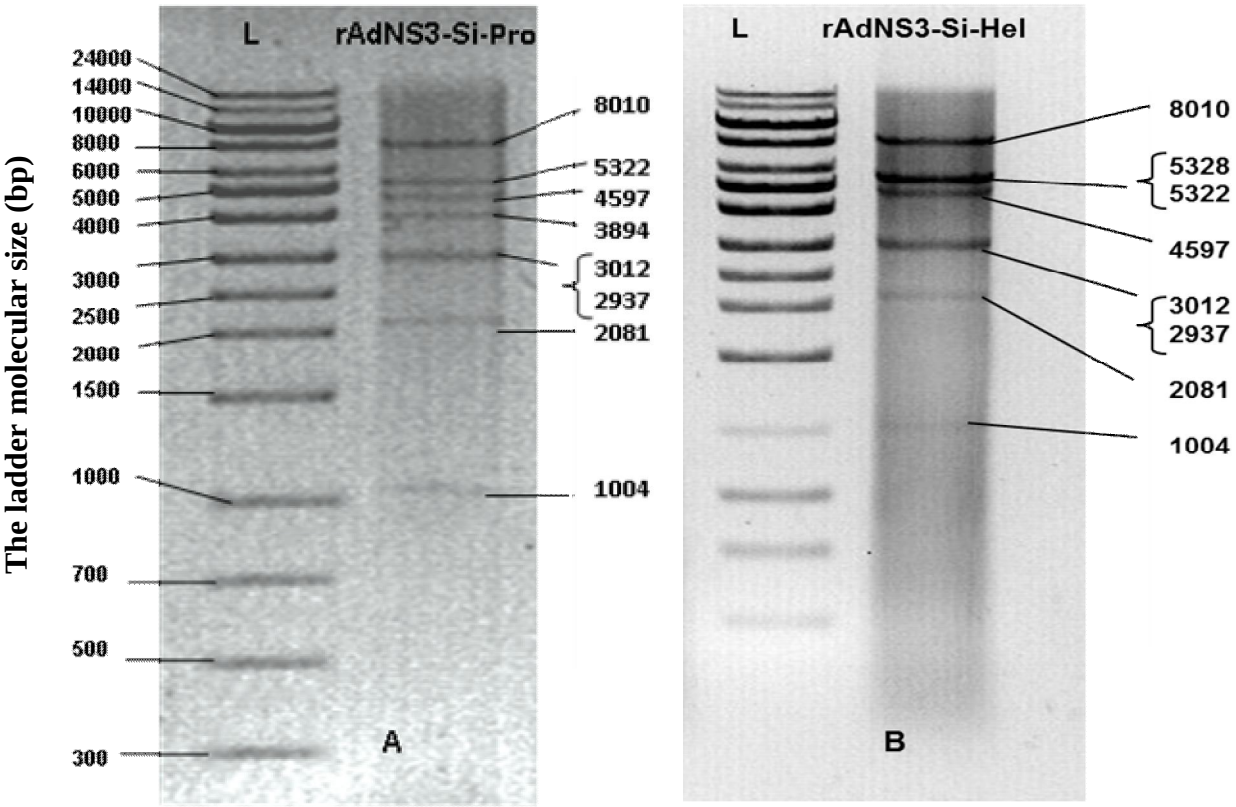


Figure 4.7: Confirmation of rAd-shRNA vectors. The agarose gels show the rAdNS3-Si-Pro (A) and rAdNS3-Si-Hel (B) digested with *HindIII.* The bands were as expected as shown in the table 5.1. L: Norgen's UltraRanger ladder. The sizes of ladder bands are shown in bp.

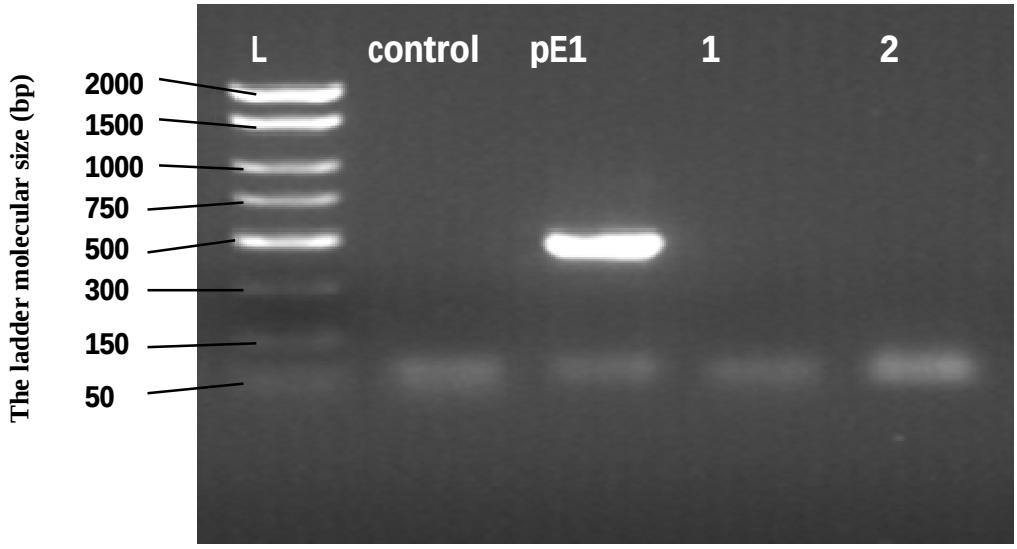


Figure 4.8: PCR amplification of AdE1 from rAdNS3-Si-Pro (lane 1) and rAdNS3-Si-Hel (lane 2). The plasmid encoding E1 (pE1) was used as the +ve control (479 bp band of E1 was obtained). E1 showed no amplification with either Ad vectors. Control: no template. L: Norgen FastRunner marker. The sizes of the ladder bands are shown in bp.

4.3.4- Down regulation of NS3 with recombinant rAd-shRNAs

The silencing activity of the rAd-shRNAs against the HCV-NS3 gene was evaluated in Con1/Fl-Neo cells, containing the HCV RNA replicon. The cells were infected with the rAdNS3-Si-Pro targeting protease and rAdNS3-Si-Hel targeting helicase at an MOI of 5. The level of NS3 mRNA was quantified at 48 h post-infection by qRT-PCR. The results showed siRNA against protease was more effective. It suppressed the expression of NS3 by 90%. The reduction in NS3 expression was statistically significant ($p < 0.05$). However, siRNA against helicase resulted in a non statistically significant level ($p > 0.05$). The results are shown in Figure 4.9.

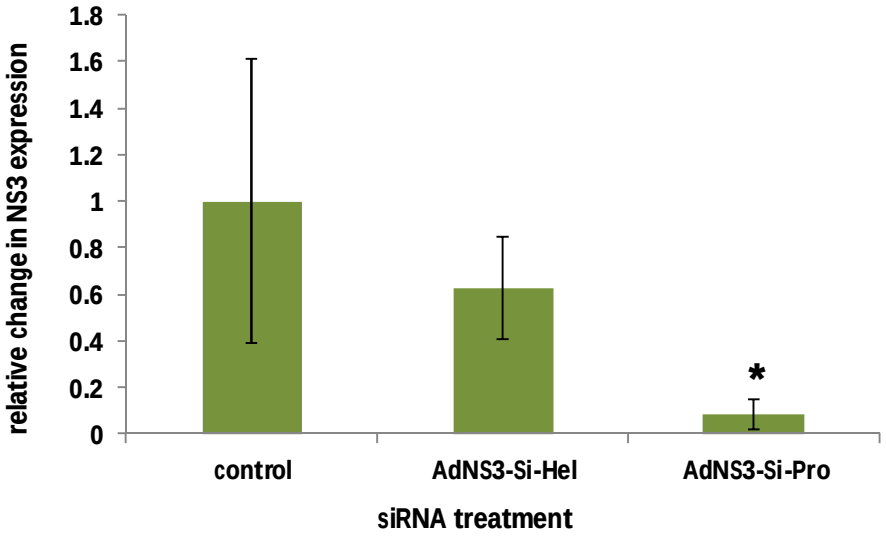


Figure 4.9: The effect of shRNA on the mRNA level of NS3 after infection of Con/FL-Neo cells with rAdNS3-Si-Hel and rAdNS3-Si-Pro. The NS3 level was expressed as a change in fold of NS3 expression compared to the control cells (not infected) measured by ΔΔCt method. Results were normalised against the S15 gene. Error bars represent the standard deviations, (n=3). Results are considered significant (*****) if p value < 0.05 as shown by T-test.

4.4- Discussion

RNAi activity against viruses showed promising results suggesting a potential application of siRNA in antiviral therapy. HCV replication and persistence in the cell was found to be dependent on critical viral proteins, such as helicase and protease, encoded by NS3, and polymerase, encoded by NS5B (Suzuki, 2007). In this study, NS3 was used as a target for siRNA, due to its highly conserved sequence among the sub genotypes. That ensures the perfect complementation required for efficient cleavage. Although the 5'UTR region is the most conserved part of the HCV genome, and it contains the IRES responsible for initiating cap-independent translation of the virus, the secondary structures and the multiple sites involved in RNA–protein interaction within the 5'UTR may protect the target sequence from recognition by siRNA. Thus, NS3 seems to be an ideal target and more effective in inhibiting the replication of HCV. shRNA expressing plasmids against NS3 showed inhibition of the expression and viral replication (Eisa, 2010; Takigawa *et al.*, 2004). Other targets such as the core, 5'UTR, NS5b was also tested using shRNAs expressed by plasmids or lentivirus vectors. These results showed that NS3 and NS5b were more effective Targets. In addition the lentivirus induced prolonged effect compared to the plasmid (Takigawa et al., 2004).

In the current study, two shRNAs sequences targeting the NS3 region were tested. Ad vectors were used for efficient siRNA delivery. NS3 encodes two essential enzymes for viral replication, protease and helicase. Two rAd-shRNAs against both the protease and helicase were constructed and tested on Con1/FL-Neo cells. The results showed that the shRNA sequence expressed by rAdNS3-Si-Pro was capable of significantly inhibiting the expression of NS3 (to 90% less than non-infected cells) (Figure 4.9). This high

inhibition may be due to the degradation of HCV genomic RNA as the siRNA sequence was designed with 100% homology to the target site. This would also indicate the integrity and absence of mutations in the target site. Ad virus showed high inhibition of NS3, which agreed with Sakamoto *et al.*, (2008) who evaluated Ad vector mediated shRNA against different target (5'UTR). siRNA against protease transcribed by the CMV ie promoter showed high activity similar to that obtained by Takigawa *et al.* (2004), who used a similar sequence under the control of the U6 promoter. The U6 promoter is responsible for the transcription of short RNA by polymerase III, and it is commonly used for shRNA transcription cassette construction. The results indicated that the CMV ie promoter can optimally transcribe shRNAs. This would be useful for delivery of antigens and shRNA sequences in only one transcription cassette.

rAdNS3-Si-Hel displayed little inhibition with no statistical significance, possibly due to a point mutation or secondary structure at the target site which hampered the access of the siRNA strand (Eisa, 2010). In addition, it was observed that siRNA target sequences are not all effective in gene silencing, and thus several sequences have to be tested to reach the optimal targets (Reynolds *et al.*, 2004). The siRNA against helicase was transcribed by U6. Since U6 is an ideal promoter for short RNA transcription, the low activity is clearly due to the shRNA sequence itself, and not the promoter. Regardless, testing several siRNAs and targeting more than one sequence in the same gene may be beneficial in gene suppression and control of escape mutants.

Finally, this study provides a proof of principle for using Ad vectors as an effective siRNA delivery strategy to suppress HCV proliferation, regarding siRNA stability, cellular uptake and cellular targeting due to their broad tropism. The strong

suppression effect of rAd-shRNA against the protease, suggests that this strategy may be effective in inhibiting viral proliferation *in vivo* since protease is a key factor in polyprotein processing and viral replication. Additionally, it would be beneficial to use several shRNAs targeting different regions of the HCV genome to further enhance the inhibitory activity.

4.5. Conclusion

The conclusion from this chapter is as follows:

1- Two Ad vectors carrying shRNA against HCV-NS3 were constructed, one is directed against the protease (rAdNS3-Si-Pro) and the other against the helicase (rAdNS3-Si-Hel).

2- rAdNS3-Si-Pro induced aproximately 90% reduction in the level of NS3 mRNA.

3- rAdNS3-Si-Hel induced only a non-significant reduction in NS3 mRNA.

4- Not all siRNAs are optimal for supressing gene expression.

5- The CMV ie promoter displayed optimal activity that is comparable to the U6 promoter in transcription of shRNAs.

6- Ad vectors were shown to be promising candidate for efficient shRNA delivery.

GENERAL CONCLUSION

In this thesis, three approaches for vaccination and therapy against HCV were investigated. These include using alternative promoters and viral delivery aiming to optimize HCV vaccine efficacy as well as using siRNA to control the virus replication as a therapeutic approach.

In the first strategy, the effect of various promoters on the efficacy of DNA vaccines was evaluated, more specifically, the weak but immediate early promoter E1A, the strong but late promoter MLP. These promoters were compared to the standard CMV ie promoter. In the animal model tested, the CMV ie promoter appears to be more effective than the other promoters tested.

In the second strategy, an adenovirus vector expressing the HCV-NS3 gene was constructed. The data demonstrated a high antibody titer level upon injection of the mice due to the efficient gene delivery and high levels of gene expression. A durable immune response was observed six weeks after single dose administration. Moreover, miRNAs, indicative of a strong immune response, were detected**.** mir-181 and mir-21 were up-regulated after immunization with the Ad vector, indicating a correlation between the miRNA level and the immune response elicited by Ad.

In the third strategy, siRNAs against the HCV protease and helicase were expressed by Ad vectors. The data indicated that the siRNA sequence against protease could successfully target the mRNA of NS3 for inhibition, indicating that RNAi would be an effective antiviral therapy. In addition, target choice and delivery method are critical

for effective RNAi therapy. Ad vectors have been shown to be a potent delivery method for siRNA.

In summary, the results showed that promoter strength and delivery method have an important impact on vaccine outcome. Moreover, siRNA can effectively inhibit gene expression of HCV. The Ad vaccine encoding NS3 and E1 is a candidate vaccine, capable of inducing a high and durable cross reactive immune response. However, small animal models are not ideal for evaluation of DNA vaccines. Therefore, testing in a large animal model is necessary. Also, Ad-encoding shRNA against protease could be a successful therapeutic approach to clear the HCV infection. The elevated level of miRNAs after vaccination,, indicates their role in immune response regulation. This suggests that miRNAs could be novel regulators to enhance vaccine efficacy as well as measuring tools to evaluate the immune response induced by vaccines.

APPENDIX

```
   1 tatgaagtgc gcaacgtatc cggagtgtac catgtcacga acgactgctc caacgcaagc
  61 attgtgtatg aggcagcgga catgatcatg catacccccg ggtgcgtgcc ctgcgttcgg
 121 gagaacaact cctcccgctg ctgggtagcg ctcactccca cgctcgcggc caggaacgct
 181 agcgtcccca ctacgacgat acgacgccat gtcgatttgc tcgttggggc ggctgctctc
 241 tgctccgcta tgtacgtggg agatctctgc ggatctgttt tcctcgtcgc ccagctgttc
 301 accttctcgc ctcgccggca cgagacagta caggactgca attgctcaat atatcccggc
 361 cacgtgacag gtcaccgtat ggcttgggat atgatgatga actggtcacc tacagcagcc
 421 ctagtggtat cgcagttact ccggatc
```

Figure A.1: The sequence of HCV-E1 cDNA (truncated) (447 bp).

```
   1 gcgcctatta cggcctactc ccaacagacg cgaggcctac ttggctgcat catcactagc
  61 ctcacaggcc gggacaggaa ccaggtcgag ggggaggtcc aagtggtctc caccgcaaca
 121 caatctttcc tggcgacctg cgtcaatggc gtgtgttgga ctgtctatca tggtgccggc
 181 tcaaagaccc ttgccggccc aaagggccca atcacccaaa tgtacaccaa tgtggaccag
 241 gacctcgtcg gctggcaagc gccccccggg gcgcgttcct tgacaccatg cacctgcggc
 301 agctcggacc tttacttggt cacgaggcat gccgatgtca ttccggtgcg ccggcggggc
 361 gacagcaggg ggagcctact ctcccccagg cccgtctcct acttgaaggg ctcttcgggc
 421 ggtccactgc tctgcccctc ggggcacgct gtgggcatct ttcgggctgc cgtgtgcacc
 481 cgaggggttg cgaaggcggt ggactttgta cccgtcgagt ctatggaaac cactatgcgg
 541 tccccggtct tcacggacaa ctcgtcccct ccggccgtac cgcagacatt ccaggtggcc
 601 catctacacg cccctactgg tagcggcaag agcactaagg tgccggctgc gtatgcagcc
 661 caagggtata aggtgcttgt cctgaacccg tccgtcgccg ccaccctagg tttcggggcg
 721 tatatgtcta aggcacatgg tatcgaccct aacatcagaa ccggggtaag gaccatcacc
 781 acgggtgccc ccatcacgta ctccacctat ggcaagtttc ttgccgacgg tggttgctct
 841 gggggcgcct atgacatcat aatatgtgat gagtgccact caactgactc gaccactatc
 901 ctgggcatcg gcacagtcct ggaccaagcg gagacggctg gagcgcgact cgtcgtgctc
 961 gccaccgcta cgcctccggg atcggtcacc gtgccacatc caaacatcga ggaggtggct
1021 ctgtccagca ctggagaaat ccccttttat ggcaaagcca tccccatcga gaccatcaag
1081 ggggggaggc acctcatttt ctgccattcc aagaagaaat gtgatgagct cgccgcgaag
1141 ctgtccggcc tcggactcaa tgctgtagca tattaccggg gccttgatgt atccgtcata
1201 ccaactagcg gagacgtcat tgtcgtagca acggacgctc taatgacggg ctttaccggc
1261 gatttcgact cagtgatcga ctgcaataca tgtgtcaccc agacagtcga cttcagcctg
1321 gacccgacct tcaccattga gacgacgacc gtgccacaag acgcggtgtc acgctcgcag
1381 cggcgaggca ggactggtag gggcaggatg ggcatttaca ggtttgtgac tccaggagaa
1441 cggccctcgg gcatgttcga ttcctcggtt ctgtgcgagt gctatgacgc gggctgtgct
1501 tggtacgagc tcacgcccgc cgagacctca gttaggttgc gggcttacct aaacacacca
1561 gggttgcccg tctgccagga ccatctggag ttctgggaga gcgtctttac aggcctcacc
1621 cacatagacg cccatttctt gtcccagact aagcaggcag gagacaactt cccctacctg
1681 gtagcatacc aggctacggt gtgcgccagg gctcaggctc cacctccatc gtgggaccaa
1741 atgtggaagt gtctcatacg gctaaagcct acgctgcacg ggccaacgcc cctgctgtat
1801 aggctgggag ccgttcaaaa cgaggttact accacacacc ccataaccaa atacatcatg
1861 gcatgcatgt cggctgacct ggaggtcgtc acg
```

Figure A.2: The sequence of HCV-NS3 cDNA (1893 bp).

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